

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of  
Linda D. MARTIN *et al.*  
Serial No. 10/802,644  
Filed: March 17, 2004  
For: BLOCKING PEPTIDE FOR INFLAMMATORY CELL SECRETION



Atty. Docket No: 5051-574P  
Group Art Unit: 1644  
Examiner: Maher M. Haddad

DECLARATION UNDER 37 C.F.R. §1.132

I, Kenneth B. Adler, declare that:

1. I am one of the inventors of the above-referenced application. I have held the position of Professor in the Department of Molecular Biomedical Sciences, North Carolina State University, College of Veterinary Medicine, Raleigh, North Carolina since July 1987. I am a cell biologist and have worked exclusively in the respiratory field with a particular emphasis on airway pathology and mucin secretion from the early 1980's to the present. A copy of my Curriculum Vitae is appended hereto as Exhibit 1.

2. I have read and understood the rejections based on the alleged lack of written description, alleged lack of enablement and alleged non-obviousness of the claimed invention in the Office Action in the above-captioned application, mailed on June 29, 2005. In response to these rejections, I provide the following comments and clarifications supported by scientific publications where appropriate.

3. More specifically, the Examiner alleged that an abstract, of which I am a co-author, published in CHEST 2000: 117:266S-267S, ("the Adler abstract") makes it obvious to one of ordinary skill in the art at the time of the invention to inhibit the release of an inflammatory mediator in a subject by administering a therapeutically effective amount of a MANS peptide (SEQ ID NO:1). The Examiner's rejection appears to be relying on the first sentence of this abstract that recites "[h]ypersecretion of mucus contributes to airway inflammation and obstruction in COPD." Firstly, this sentence was not intended to establish or support a direct scientific link between mucus secretion and

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inflammation. What I intended to convey by this sentence is that excess mucus in the airways makes the individual more susceptible to microbial infection, which then can possibly result in inflammation that is caused by the microbial infection. There is no DIRECT link between excess mucus and inflammation. Although inflammation in the airways leads to excess mucus production and secretion, the inverse is not true. Mucus secretion can be stimulated by a number of factors, including mediators released by inflammatory cells, i.e., granulocytes, which include neutrophils, basophils and eosinophils. Although some inflammatory mediators from many different cells can affect secretion, secretion of mucus and inflammation are two separate processes. With all of my years experience in this field, I am not aware of any evidence in the scientific literature or any information at scientific meetings nor have I ever observed in my own experiments that mucus secretion directly results in inflammation.

4. The Adler abstract does not suggest to a person of ordinary skill in the art, such as myself, that it would be obvious to consider administering the MANS peptide to block the release of inflammatory mediators. As discussed above, the skilled person would know that mucus secretion does not cause airway inflammation. Therefore, there would be no reason to believe that administering MANS peptide would block inflammatory mediator release from inflammatory cells.

5. In support of my position in paragraphs 3 and 4 above, I enclose a copy of a publication by Haile *et al.* published in 1999 before the filing date of the priority document of the present application which is attached as Exhibit 2. This publication is entitled "Mucus-Cell Metaplasia and Inflammatory-Cell Recruitment Are Dissociated in Allergic Mice after Antibody- and Drug-Dependent Cell Depletion in a Murine Model of Asthma," and by its title supports my position that mucus secretion is disassociated from inflammation. I direct the Examiner's attention to the abstract which summarizes the results of this research. Specifically, lines 12-16 of the abstract, recite that "[t]reatment with the granulocytopenic drug vinblastine before challenge completely abolished the recruitment of granulocytes without affecting the antigen-induced mucous-cell metaplasia." Haile *et al.* conclude that mucus secretion is independent of the presence of

granulocytes and neutrophils are a type of granulocyte. (page 892, 1<sup>st</sup> column, last sentence of bridging paragraph) It is my opinion that this publication shows that inflammation, as defined by the influx of granulocytes, i.e., neutrophils, is clearly not associated with mucus cell metaplasia.

6. In response to the Examiner's statement that the *in vitro* experiments presented in my patent application do not correlate to a practical *in vivo* use, I would like to explain that mechanisms for all inflammatory processes comprise (a) chemotactic migration of inflammatory cells to a site of injury in a tissue, and (b) release of inflammatory mediators from the inflammatory cell. The primary inflammatory cells include granulocytes, which are neutrophils, eosinophils, and basophils. An assay comprising an actual count of the number of migrated inflammatory cells and an assay comprising a quantitative determination of the amounts of inflammatory mediators released at the injured tissue site can be used to assess the degree of inflammation in tissue injured in a particular disease. In practice, inflammatory mediators such as myeloperoxidase (MPO), elastase, lysozyme and others are quantitated using specific biochemical assays.

7. One or both of the above assays are routinely used to determine the inflammatory capacity of an agent or to determine the agent's property as an inhibitor of inflammation. The second assay uses isolated blood cells for quantitative determination of release of inflammatory mediators mediated by an agonist or inhibition of release of inflammatory mediators by an antagonist. It is my opinion that these assays are very predictive of *in vivo* situations, and therefore they are routinely used in place of animal models irrespective of the site or tissue where the inflammation has occurred. Therefore, the data presented in Figs. 9-15 show that MPO can be accurately measured *in vitro* when inhibited and stimulated by various agents. Specifically, Figs. 9 and 10 show that MPO is inhibited in both human and canine neutrophils by MANS peptide in a dose-dependent manner. It is further my opinion as a person skilled in that art that these *in vitro* studies are predictive of the outcome *in vivo*.

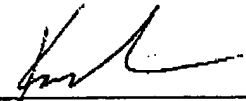
8. In further support of my position that studies using isolated granulocytes are predictive *in vitro* models for studying their inflammatory function *in vivo*, I enclose a publication by Abdel-Latif *et al.* (Exhibit 3) showing that neutrophils isolated from mice with a targeted gene deletion maintain this deletion *in vitro*, and thus function as they would *in vivo*. This publication reports on the use of these isolated neutrophils to investigate degranulation responses to chemoattractant stimulation. The first, second and third complete paragraphs in the first column on page 833 support this statement with the second paragraph showing that neutrophils isolated from a human patient, who essentially had the same genetic defect as was generated in the mice, also maintained this defect when isolated and studied in an *in vitro* system similar to the system disclosed in the present application. Additionally, the last sentence of the second column on page 838 concludes that these *in vivo* studies using isolated granulocytes provide an accepted means for developing targets for anti-inflammatory therapies.

9. In further support of my position, enclosed is another publication by Lacy *et. al.* (Exhibit 4), showing that isolated granulocytes from asthmatic patients responded differently *in vitro*, mimicking their *in vivo* aberrations. The last paragraph of the discussion, page 2677, and particularly the last sentence of this paragraph states that the *in vitro* studies with the eosinophils and neutrophils are important in studying cell activation and ultimately treatment. Thus, these *in vitro* uses of granulocytes support my position that *in vitro* studies are predictive of *in vivo* studies, and these publications show that other scientists in the inflammatory field rely on the results generated from these *in vitro* studies to predict the outcome in *in vivo* studies.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001

of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

10/31/85  
Date

  
Kenneth B. Adler, Ph.D.

257310 v1/RE



Attorney Docket No: 5051.574ct

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Martin et al.

Confirmation No.: 3963

Application No.: 10/802,644

Examiner: Maher M. Haddad

Filed: March 17, 2004

Group Art Unit: 1644

For: *Blocking Peptide for Inflammatory Cell Secretion*

Date: October 31, 2005

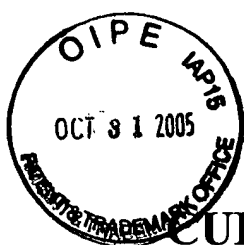
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Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

**Exhibit 1**



## CURRICULUM VITAE

KENNETH BRUCE ADLER, Ph.D.

October, 2005

### Home Address

5301 Wood Valley Drive  
Raleigh, NC 27613  
(919) 847-1765

### Work Address

College of Veterinary Medicine  
North Carolina State University  
4700 Hillsborough St.  
Raleigh, NC 27606  
Office: (919) 513 1348  
Lab: (919) 513 1347  
FAX: (919) 515 4237  
E-MAIL: kenneth\_adler@ncsu.edu

### EDUCATION

Ph.D.- Cell Biology, University of  
Vermont, Burlington, VT, 1978  
M.S. - Biology, Adelphi University  
Garden City, NY, 1975  
B.S. - Biology, Queens College,  
Flushing, NY, 1969

### ACADEMIC APPOINTMENTS

Professor, Department of Molecular Biomedical Sciences, North Carolina State University, College of Veterinary Medicine, Raleigh, North Carolina, July 1990 -

Associate Professor, Department of Anatomy, Physiological Sciences and Radiology, North Carolina State University, College of Veterinary Medicine, Raleigh, North Carolina, July 1987 - June 1990

Assistant Professor, Department of Pathology, University of Vermont College of Medicine, Burlington, VT, July 1984 - June 1987.

Research Assistant Professor, Department of Pathology, University of Vermont College of Medicine, Burlington, VT, July 1979 - June 1984.

Research Associate/Graduate Student, Department of Pathology, University of Vermont College of Medicine, Burlington, VT, July 1975 - June 1979.

Research Biologist, Veterans Administration Hospital, Brooklyn, NY, 1973 - 1975.

Biophysicist, Veterans Administration Hospital, Brooklyn, NY, 1969 - 1972.

### HONORS AND AWARDS

**CURRICULUM VITAE**  
**KENNETH B. ADLER, Ph.D.**  
**Page 2**

**NIH NHLBI MERIT Award (5 R37 HL-36982): 2004 - 2014**

**The Oliver Max Gardner award for Service to Humanity**, University of North Carolina System, 2005

**Alexander Quarles Holladay Medal for Excellence ("the highest award made by the university in recognition of faculty career accomplishments")**; North Carolina State University, 2004.

**Alumni Association Award for Research Excellence**, North Carolina State University, 1999.

**Alumni Distinguished Graduate Professor**, North Carolina State University, 1998-2000.

**Established Investigator**, American Heart Association, 1987 - 1992.

**Smith, Kline and Beecham Award for Research Excellence**, North Carolina State University College of Veterinary Medicine, 1991.

**The Ruth L. and Ned E. Huffman Leadership Award**, North Carolina State University College of Veterinary Medicine, 1996.

**Postdoctoral Fellow**, American Lung Association, 1978 - 1980.

**EDITORIAL BOARDS and COUNCILS:**

American Journal of Respiratory Cell & Molecular Biology;

**DEPUTY EDITOR: 2003 -**

**ASSOCIATE EDITOR: 2003 -**

**EDITORIAL BOARD: 1994 -**

Respiratory Research; Associate Editor: 2004 -

Journal of Organ Dysfunction; Editor: 2005 -

American Journal of Physiology; Lung Cellular and Molecular Physiology; Editorial Board: 1995 -

Clinical Science; Editorial Board; 2001 -

International Journal of Biochemistry and Cell Biology; Editorial Board; 2004 -

Encyclopedia of Respiratory Medicine; Editorial Advisory Board: 2002 -

Aspen Lung Conference International Advisory Board; 1999-

Science Advisory Council, CIIT Centers for Health Research, RTP, NC. 2001, 2005

**PROFESSIONAL SOCIETIES**

American Thoracic Society: Member, Program Committee, Long Range Planning

Committee, Nominations Committee: Assembly on Respiratory Cell and Molecular Biology

American Society for Cell Biology

American Society of Investigative Pathology

Phi Zeta: The Honor Society of Veterinary Medicine

**JOURNAL REVIEWS**



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Academic Press  
American Journal of Medicine  
American Journal of Pathology  
American Journal of Physiology: Lung Cellular & Molecular Physiology  
American Journal of Physiology: Cellular Physiology  
American Journal of Respiratory Cell & Molecular Biology  
American Journal of Respiratory & Critical Care Medicine  
American Review of Respiratory Disease  
Applied Pathology (member: International Advisory Board, 1984-90)  
Archives of Biochemistry and Biophysics  
Archives of Internal Medicine  
Biochemical Journal  
Biochemical Pharmacology  
Bioinformatics  
Biotechniques  
Br J Pharmacol  
Cell and Tissue Research  
Cell Motility and the Cytoskeleton  
Chest  
Clinical Science  
CRC Publishing Co.  
Critical Reviews in Toxicology  
Critical Care Medicine  
Digestive Diseases and Sciences  
Environmental Health Perspectives  
European Journal of Respiratory Disease  
Evidence Based Complementary and Alternative Medicine  
Experimental Cell Research  
Experimental Lung Research  
FASEB Journal  
Free Radical Biology & Medicine  
Gastroenterology  
Glycoconjugate Journal  
Hepatology  
In Vitro: Cell and Developmental Biology  
Journal of Allergy and Clinical Immunology  
Journal of Applied Physiology  
Journal of Cell Biology  
Journal of Cell Science  
Journal of Cellular Biochemistry  
Journal of Cellular Physiology  
Journal of Clinical Investigation

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Journal of Histochemistry and Cytochemistry  
Journal of Immunology  
Journal of Laboratory and Clinical Medicine  
Journal of Molecular Medicine  
Journal of Pharmacology & Experimental Therapeutics  
Journal of Veterinary Pharmacology & Therapeutics  
Journal of Physiology  
Journal of Virology  
Laboratory Investigation  
Life Sciences  
Pediatric Research  
Pharmaceutical Research  
Physiological Reviews  
Proceedings of National Academy of Science - USA  
Proceedings Society Experimental Biology and Medicine  
Pulmonary Pharmacology  
Pulmonary Pharmacology & Therapeutics Lung  
Respiratory Research  
Respiratory Physiology & Neurobiology  
Scanning Electron Microscopy  
Thorax  
Toxicology and Applied Pharmacology

**RESEARCH INTERESTS**

- 1) Airway inflammation and mucus production
- 2) Signal transduction
- 3) Epithelial cell culture

**RESEARCH SUPPORT**

**CURRENT:**

Principal Investigator:

NIH NHLBI 5 R37 (MERIT AWARD) HL 36982 "Mechanism of Oxidant-Induced Respiratory Mucin Secretion." (7/01/86 - 2/29/14). Total; \$7,263,153.00 (approx.)

U.S. EPA CT826512010 "Cooperative Training in Environmental Sciences Research". (8/01/01-7/31/06). Total; \$ 3,206,219.00 (approx.).

AstraZeneca Inc., Lund, Sweden. "Anti-mucin and anti-inflammatory effects of budesonide + formoterol combination on primary cultures of human airway epithelial cells". (06/01/04 – 5/30/05). Total; \$70,000.00

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North Carolina Biotechnology Center, RTP, NC: "A Novel Approach for Treatment of Airway Mucus Hypersecretion" (07/01/03 – 06/30/05). Total: \$120,000.00

Sepracor Corporation, Marlborough, MA. "Differential Effects of Albuterol Isomers on Human Airway Epithelium-Smooth Muscle Interactions" (Supplement 06/01/04-12/31/04). Total; \$20,000.00

GlaxoSmithKline Corporation (1/93 - indefinite) "Effects of Oxidants and TNF $\alpha$  on NF-kB and ICAM-1 Expression on Airway Epithelium" Total; \$46,980.00

Hoffmann La Roche Corporation (7/85 - indefinite) "Airway Epithelial Function". Total; \$38,000.00

**PENDING:**

None

**PAST:**

Principal Investigator:

Established Investigator; American Heart Association (7/87 - 6/92). "Platelet Activating Factor and Airway Epithelium". Total; \$175,000. (Stipend).

NIH NIEHS 1T32 ES07311 "Environmental Cellular & Molecular Pathology". (7/01/99 - 6/30/04). Total; \$783,456.00

NIH NIEHS 1F32ES11245 "Mechanisms of Diesel-Enhanced Allergic Sensitization". (10/1/01-3/30/04). NRSA co-sponsor for Pramila Singh, Ph.D. Total \$116,079.00

Schering Corporation, Kenilworth, NJ. "Mucin Gene Expression in an Animal Model of Asthma" (7/01/99 - 6/30/01). Total; \$238,361.00

Sepracor Corporation, Marlborough, MA. "Differential Effects of Albuterol Isomers on Human Airway Epithelium-Smooth Muscle Interactions" (10/01/01-09/30/02). Total; \$38,305.00

U.S. EPA CT826512010 "Cooperative Training in Environmental Sciences Research". (4/01/98-3/31/01). Total; \$1,682,393.00

U.S. Environmental Protection Agency C R 824355-01-0 (10/01/95 - 9/30/97) "Effects of Pollutant Dusts on Airway Epithelium." Total; \$49,760.

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NIH NHLBI F3209689 "Mechanisms of IL-6 Gene Expression in Airway Epithelium". (4/01/97 - 3/31/00). NRSA Sponsor for Linda D. Martin, Ph.D. Total; \$107,500.

NIH NIEHS/NHLBI 1R13ES08989-01 "Second International Meeting on Oxygen/Nitrogen Radicals and Cellular Injury" (9/6 - 9/10. 1997). Total; \$10,000.

NIH NHLBI F32HL09512 "Mechanisms of TNF $\alpha$  Induced ICAM-1 in Airway Epithelium". (4/01/96 -3/31/99). NRSA Sponsor for Thomas M. Krunkosky, DVM. Total; \$107,500.

NIH NHLBI "Effects of TNF $\alpha$  on Airway Epithelium". (10/01/94 -9/30/97). NRSA Sponsor for Bernard Fischer, DVM. Total; \$107,500.

NIH NHLBI 1 RO1 HL 37636 (7/87 - 6/91). "Platelet-Activating Factor and Respiratory Mucin Secretion." Total; \$287,237.

Biomarck Corporation, RTP, NC "Studies on Airway Mucin Secretion" (08/01/03 – 07/31/04). Principal Investigator: Neil C Olson, D.V.M., Ph.D. Role in Project: Co-Investigator. Total: \$86,000.00

Schering Corporation, Kenilworth, NJ: "Development of Molecular Probes against Guinea Pig Airway Mucins." (2/97-9/98). Total \$71, 277.

NIH NHBLI 14212 (12/86 - 11/91). "SCOR in Occupational and Immunologic Lung Disease." Director, Morphology Core (Core F). Total; \$359,806. (Consultant as of 7/1/87).

Cystic Fibrosis Foundation, "Lesions of Cystic Fibrosis and Prostaglandins." (10/84-2/87). Total; \$60,000.

North Carolina State University, School of Veterinary Medicine (7/1/87-6/30/88) "Morphometric Characterization of a novel system for maintaining rodent respiratory epithelial cells in primary culture." Total; \$8,000.

North Carolina State University, College of Veterinary Medicine (7/1/91-6/30/92) "Generation of reactive oxygen species by airway epithelium: Effects of neutrophil-derived products" total; \$24,100.

North Carolina State University, College of Veterinary Medicine (7/1/92-6/30/93) "Oxidant-Antioxidant Function of Airway Epithelium" total; \$23,885.

North Carolina State University, College of Veterinary Medicine (7/1/93-6/30/94) "Effects of Oxidant Stress on Airway Epithelial Cells" total; \$24,661.

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North Carolina State University, College of Veterinary Medicine (7/1/95-6/30/96) "Effects of TNF $\alpha$  on Expression of ICAM on Airway Epithelium" Total; \$24,838.

North Carolina State University, College of Veterinary Medicine (7/1/96-6/30/97) "Mechanisms of Nitric Oxide production by airway epithelial cells" Total; \$24,798.

North Carolina State University College of Veterinary Medicine "Molecular Mechanisms Governing ROS-Mediated Gene Expression in Airway Epithelium." (7/1/98-6/30/99). Total \$14,943

Council for Tobacco Research USA, Inc. "Airway Mucin Secretion: Effects of Products From Bacteria Associated With Chronic Bronchitis." (1/83-6/86). Total; \$215,000.

NIH NIAID/NHLBI "Effects of Pseudomonas Products on Airway Mucin Secretion. (8/81-7/84). Total; \$112,500. (New Investigator Award).

Cystic Fibrosis Foundation "Cellular Mechanisms of Normal and Hypersecretion by Respiratory Airway Tissue." (7/79-6/81). Total; \$54,000.

American Lung Association (7/78-6/80) Postdoctoral Fellowship. Total \$24,000 (Stipend).

NIH NIEHS 7 F32 ES05547 "Mechanisms of asbestos-induced injury to alveolar epithelium ". (7/01/91-5/30/95). NRSA Sponsor for Sarah Gardner, DVM. Total; \$124,500.

NIH NHLBI "Oxidant-antioxidant function of airway epithelium." (7/01/92-6/30/95). NRSA Sponsor for Leah A. Cohn, DVM. Total; \$107,500.

**Co-Investigator:**

North Carolina Biotechnology Center, RTP, NC. "Transfection of Differentiated Human Airway Epithelial Cells in Vitro: Cell-Specific and Inflammatory Responses to Gene Therapies". PI: Linda D. Martin, Ph.D. (9/01/99 - 2/28/01). Total; \$40,000.

NIH NHLBI "Effects of hypoxia on pulmonary vasculature". (7/87- 6/92). Dr. J. Evans, PI. Total; \$611,635.

NIH NHLBI "Single vascular smooth muscle mechanics".(9/85-8/90). Dr. D. Warshaw, PI. Total; \$760,663.

NIH NIEHS "Role of metallothionenes in pulmonary injury". (3/87- 2/90). Dr. B. Hart, PI. Total; \$312,673.

NIH NHLBI 14212 "Correlated Studies of Pulmonary Fibrosis (SCOR). 12/76-11/86). Dr. R. Low,

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SCOR Director. Total; approx. \$8,000,000.

NIH NHLBI "Respiratory Cytopathology of Mt. St. Helens' Dust". (4/81-3/84). Dr. J. Craighead, PI. Total; \$214,677.

NIH NHLBI "Micromechanics and Biochemistry of Pulmonary Parenchyma". (9/81-8/84). Dr. J. Evans, PI. Total; \$221,365.

NIH NHLBI "Mechanics of intrapulmonary airway smooth muscle".(10/85 - 9/88). Dr. J Evans, PI. Total; \$271,580.

**STUDY SECTIONS AND REVIEW COMMITTEES:**

PERMANENT MEMBER; NIH Lung Biology and Pathology (LBPA) Study Section,  
1991-1994  
1999-2004

**CHAIR: 2002-2004: NIH Lung Cell & Molecular Immunology (LCMI) Study Section**

PERMANENT MEMBER; American Lung Association/ American Thoracic Society, Research  
Review Study Section 1995 - 1998  
American Lung Association Asthma Center Study Section, 1996

PERMANENT MEMBER; Veterans Administration Respiration Merit Review  
Board, 1988-1991

PERMANENT MEMBER; California Tobacco-Related Disease Research  
Program; Pulmonary Study Section, 1992 - 1994, 1997, 1999, 2000, 2001, 2003, 2004,  
2005

PERMANENT MEMBER; State of Nebraska: Cancer and Smoking Disease Research Program,  
Technical review Panel, 1995 - present  
**CHAIR: 1999 – 2002**

MEMBER; CIIT Centers for Health Research Science Advisory Committee, 2001, 2005

Other Study Sections;

NIH Pathology A Study Section, 1988, 1990

NIH Respiratory & Applied Physiology Study Section, 1988, 1990, 1995, 1996, 1997, 1998, 1999

NIH Physiological Sciences Study Section, 1995, 1996, 1997, 1998, 1999

NIH Lung Biology & Pathology Study Section, 1995, 1996, 1997, 1998, 1999

NIH Special Emphasis Panels, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005

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NIH Program Project and SCOR proposals, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005

Reviewer and site visit participant:

American Institute of Biological Sciences  
Canadian Cystic Fibrosis Foundation  
Veterans Administration Medical Research Council  
Council for Tobacco Research, USA, Inc.  
Environmental Protection Agency  
Medical Research Council of Canada  
United States Dept. of the Interior; Bureau of Mines.  
Health Effects Institute  
U.S. Dept. of Defense  
National Cancer Institute  
United States Department of Agriculture  
National Heart, Lung and Blood Institute  
National Institute of Environmental Health Sciences  
National Institute of Diabetes, Digestive & Kidney Diseases  
State of Louisiana Board of Regents  
Louisiana Cancer and Lung Trust fund Board  
The Finn Foundation  
United States Department of Agriculture  
Cystic Fibrosis Research, Inc.  
The Wellcome Trust  
British Lung Foundation  
The Thrasher Foundation  
March of Dimes Birth Defects Foundation  
James and Esther King Biomedical Research Program: Florida Department of Health  
Health Research Board Ireland  
The Nanotechnology Institute (NTI) of Pennsylvania

**Teaching Experience:**

North Carolina State University

Chair, Cell Biology/Morphology Concentration Area, CBS Graduate Program, 1987 - 2000.

Graduate Course Coordinator: CBS 770; Recent Advances in Cell Biology, 1989 - 2001  
Approximately 12 lecture hrs. per year.

Graduate Course Coordinator: CBS 807; Seminar in Cell Biology, 1989 - 2001

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Graduate Course Coordinator: VMS 590L; Environmental Cell Biology (Spring 1992)

CBS 810: "Grant writing and other practical aspects of a scientific career: (Spring, 2003)

University of Vermont

Lecturer, Cell Biology 301; Approximately 5 lecture hrs. per year, 1980 - 1986.

Lecturer, Cell Biology 302; 1 lecture hr. per year, 1986.

**Graduate Students and Fellows Supervised:**

GRADUATE STUDENTS

University of Vermont

Gregory Butler: Ph.D. 1985. Presently Attorney-at-Law, Boston, MA.

Linda Callahan: M.S. 1985. Presently Research Assistant Professor, University of Rochester

North Carolina State University

Lynn Blalock: Ph.D. 1991. Presently Staff Scientist, U.S. EPA, RTP, NC

\*Leah A. Cohn, DVM: Ph.D. 1994. Presently, Associate Professor, University of Missouri College of Veterinary Medicine, Columbia, MO.

Hongfei Li, MD: Ph.D., 1995. Presently Physician, Bethesda, MD.

Chengming Li, MD: Ph.D., 1995. Presently Research Associate, Duke University Medical Center, Durham, NC.

David T. Wright: Ph.D., 1994. Presently Manager, International Non Clinical Registration, Glaxo SmithKline Corp., RTP, NC.

Nonghoon Choe: Ph.D., 1995. Presently Assistant Professor, Konkuk University, College of Veterinary Medicine, Seoul, Republic of Korea.

Jianngwu Lee: Ph.D., 1995. Presently Associate Professor, College of Veterinary Medicine, National Taiwan University, Taipei, Taiwan.

\*Sarah Y. Gardner, DVM: Ph.D., 1995. Presently Associate Professor, NCSU College of Veterinary Medicine, Raleigh, NC.

\*Bernard Fischer, DVM: Ph.D. 1997, Presently Assistant Professor, Duke U., Durham, NC.

Janice Dye, DVM: Ph.D. 1998, Presently Senior Staff Scientist, U.S. EPA, RTP, NC.

Nanfei Jiang, MD: Ph.D. 1998, Presently Biosystems Staff Scientist; PerkinElmer Life Sciences; Boston, MA.

\*Thomas M. Krunkosky, DVM: Ph.D. 1999, Presently Assistant Professor, University of Georgia School of Veterinary Medicine, Athens, GA.

\*\*Derek Norford, DVM, M.S.: Ph.D. 2000, Presently Head, Laboratory Animal Department, North Carolina Central University, Durham, NC.

Limin Zhang, M.S.: Ph.D., 1999. Presently Research Scientist, BioSource, Inc., Camarillo, CA

Yuehua Li, B.S.: Ph.D. 1999. Presently Senior Scientist, Tanox Corporation, Houston, TX.



## **CURRICULUM VITAE**

### **KENNETH B. ADLER, Ph.D.**

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Karrie Brenneman, D.V.M., Ph.D. 2000. Presently Staff Scientist, IDEXX Veterinary Services, Portland, OR

Fang He, M.S.: Ph.D. 2000, Presently Staff Scientist, Axiom Biotechnologies, Inc., San Diego, CA

Susan Lankford, B.S. Ph.D., 2000. Presently Research Associate, NCSU, Raleigh, NC.

\*Georgette Hill, DVM: Ph.D 2001. Presently Staff Scientist, NIEHS, RTP, NC.

Carol Anne Pettersen, M.S., Presently Scientist, EMN Corporation, RTP, NC.

Kristi Pittman, B.S. Ph.D. 2002. Presently Medical Student, University of North Carolina, Chapel Hill, NC.

Christopher Langdale, B.S., M.S. 2003. Presently Scientist, Dynogen Corporation, Boston, MA

Brian Chorley, B.S. Ph.D. 2005. Presently Postdoctoral Fellow, NIEHS, RTP, NC

Travis Knuckles, B.S., Ph.D. 2005. Presently Postdoctoral Fellow, Lovelace Research Institute, Albuquerque, NM.

Ko Wei Lin, DVM, B.S., Ph.D. expected, 2007.

Jin-Ah Park, M.S. Ph.D. expected, 2007.

\*\*Teresa Green, B.S. Ph.D. expected 2008

\*\*Kimberly Raiford, B.S. Ph.D. expected 2007

#### **POSTDOCTORAL FELLOWS:**

Lisa Kaartinen, DVM. (1989) Presently Associate Professor, University of Helsinki, Finland.

Wendy Holden-Stauffer, DVM. (1988-1990) Presently Associate Professor, U. of Berne, Switzerland.

Lori G. Rochelle, Ph.D. (1995-1996) Presently Assistant Professor, University of North Carolina @ Chapel Hill

\*Linda D. Martin, Ph.D. (1995-1998) Presently Assistant Professor, NCSU

Mariangela Macchione, Ph.D. (1999) Presently Assistant Professor, University of Sao Pablo, Brazil

Yeuhua Li, Ph.D. (1999-2002) Presently Research Scientist II, Tanox corporation, Houston, TX.

\*Pramila Singh, Ph.D. (2001-2003) Presently Staff Scientist, U.S. EPA, Research Triangle Park, NC.

Joungjoa Park, Ph.D. (2001 - 2004) Presently Research Associate, NCSU

Shuji Takashi, M.D. (2003 –

Shijing Fang, M.D. (2003) Presently Research Associate, NCSU

\*Recipient of Individual National Research Service Award from NIH

\*\* Recipient of NHLBI Research Supplement for Underrepresented Minorities

#### **Graduate Committees**

Jody Khosla (M.S.; 1992)

Joan Wong (M.S.; 1992)

Shou Wu Wang (Ph.D.; 1993)

John Dodam (Ph.D; 1994)

Janice Allen (Ph.D.; 1995)

Catherine Baty (Ph.D. 1996)

Doug Ryu (Ph.D; 1996)

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Jill Barnes (Ph.D; 1998)  
Jia Ma (Ph.D; 2000)  
Sharon Madison (M.S. 2001)  
Brian Booth, (Ph.D. 2004)  
Mark Cesta, DVM (Ph.D expected, 2007)

**Invited Lectures and Conferences Outside Home Institution (Last 10 years)**

**2006**

Co-Organizer: American Physiological Society Symposium “Physiological Genomics and Proteomics of Lung Disease”, Key West, FL, November, 2006.

**2005**

National Institute of Environmental Health Sciences; National Center for Toxicogenomics, RTP, NC. November, 2005. Host: Dr. Kevin Gerrish

"Epithelial pathophysiology in chronic lung diseases", Invited Speaker; Shanghai International Respiratory Symposium, Shanghai, China, October, 2005 .

University of Alabama @ Birmingham, Birmingham, AL, September 2005. Host: Dr. Lori McMahon

University of Pennsylvania School of Medicine, Philadelphia, PA, August 2005. Host: Dr. Angela Haczku

“Regulation of mucus secretion in the airway epithelium”, Invited Speaker, World Equine Airways Symposium, Cornell University, Ithaca, NY, July 2005.

“Grant Fundamentals”; Invited Speaker; Postgraduate Course, American Thoracic Society Annual Meeting, San Diego, CA, May 2005.

University of Vermont College of Medicine, Burlington, VT. April, 2005. Host: Dr. Charles Irvin

“Mechanisms of mucous secretion”, Invited Speaker, plenary session entitled “New Paradigms in Upper and Lower Airways Diseases”, American Academy of Allergy, Asthma & Immunology Annual Meeting, San Antonio, Texas, March 2005.

Vice-Chair, Gordon Conference; CILIA, MUCUS, and MUCOCILIARY INTERACTIONS, Buellton, CA, February 2005.

University of Connecticut Health Center, Farmington, CT, January 2005. Host: Dr. Roger Thrall

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**2004**

University of Massachusetts, Worcester, MA, December 2004. Host: Dr. Michael Sanderson

Indiana University School of Medicine, Indianapolis, IN, October 2004. Host: Dr. David Wilkes

University of California @ Davis, October, 2004. Host: Dr. Reen Wu

CIIT Centers for Health Research, RTP, NC, August 2004, Host: Dr. James Bonner.

U.S. Environmental Protection Agency, RTP, NC, July 2004. Invited Speaker, grant writing symposium.

“Regulation of mucin secretion”, Invited Speaker, Symposium entitled: “MUCing around in the lung” American Thoracic Society Annual Meeting, Orlando, FL, May 2004.

“Signaling pathways related to airway inflammation and secretion,” Invited Speaker, Symposium entitled "Respiratory Epithelium as a Modulator of Innate Immunity and Lung Inflammation" American Thoracic Society Annual Meeting, Orlando, FL, May 2004.

“Approaches to NIH grant writing”, Invited Keynote Speaker, 4th Young Investigators' Symposium on Smooth Muscle, Epithelium and Inflammation, Durham, NC, May 2004.

“Grant writing and Grantsmanship”, Invited Speaker, Tulane-Xavier of New Orleans ARCH workshop, May, 2004. Host: Dr. Robert Blake.

Westwood UCLA Medical Center, Los Angeles, CA, April, 2004. Host: Dr. Robert Streiter

Harbor UCLA Medical Center, Los Angeles, CA, April 2004. Host: Dr. Usha Raj

National Institute of Environmental Health Sciences, RTP, NC, April 2004. Host: Dr. Anton Jetten

University of North Carolina at Chapel Hill, March, 2004. Host: Dr. Raymond Pickles.

**2003**

Cornell University, Ithaca, NY. November, 2003. Host: Dr. Michael Kotlikoff

International Advisory Board member and Invited Speaker, X Congress of the International Rhinologic Society, XXII International Symposium on the Infection and Allergy of the Nose, Seoul, Korea, October 23-26, 2003.

Case Western Reserve University, Cleveland, OH. September, 2003. Host: Dr. Serpil Erzurum

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"Differential effects of albuterol isomers on normal human bronchial epithelial cells *in vitro*"  
Invited Speaker, Sepracor Scientific Research Forum, New Orleans, LA, September, 2003.

Chair, poster-discussion "Signaling Pathways: Pulmonary Effects," American Thoracic Society  
Annual Meeting, Seattle, WA May 2003.

Chair, poster-discussion "MUC Proteins and Mucin Regulation" American Thoracic Society  
Annual Meeting, Seattle, WA May 2003.

Sepracor Corporation, Marlborough, MA. March, 2003. Host, Dr. Anthony Magnetti

Tulane University/Xavier University of New Orleans, New Orleans, LA. March, 2003. Host: Dr.  
Arnold Brody.

Invited Participant, NIH Workshop "Respiratory Disease in Agricultural Health Study," February  
3-4, 2003, NIEHS, RTP, NC.

Mayo Clinic, Scottsdale, AZ. January 2003: Host: Dr. Jamie Lee

University of Arizona College of Medicine, Tucson, AZ. January 2003. Host: Dr. Richard Robbins

**2002**

Duke University Medical Center, Durham, NC, November, 2002. Host: Dr. Rodney Folz.

"Intracellular mechanisms of airway mucin secretion". Invited Speaker, member of Organizing  
Committee/Session Chair, Fourth International Symposium on Mucus and Mucociliary  
Interactions, Miami, FL, November, 2002.

"Mechanisms of mucin secretion by human airway epithelium." Invited Speaker, 7th Congress of  
Asian Pacific Society of Respiriology, Taipei, Taiwan, October, 2002

Creighton University School of Medicine, Omaha, NE, September, 2002. Host: Dr. Devendra  
Agrawal.

University of California @ Davis, September, 2002. Heineman lecture. Host: Dr. Reen Wu.

GlaxoSmithKline symposium "COPD Pathophysiology" Invited speaker, Philadelphia, PA. June,  
2002

University of California Los Angeles, Harbor Medical Center, March, 2002. Host: Dr. Chad Oh

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Lovelace Respiratory Research Institute, Albuquerque, NM. March, 2002. Host: Dr. Johannes Tesfaigzi..

"Effects of albuterol isomers on airway epithelial function." Invited Speaker, Sepracor Scientific Research Forum, New Orleans, LA, February, 2002.

Baylor University, Houston, TX. January 2002. Host: Dr. Tony Eissa

**2001**

University of Southern California Medical Center, Los Angeles, CA. October, 2001. Host: Dr. Edward Crandall.

North Carolina Central University, Durham, NC. September, 2001. Host: Dr. Derek Norford

Icagen Corporation, RTP, NC. August, 2001 Host: Dr. Lee Robinette.

Sepracor Corporation, Marlborough, MA. July, 2001. Host: Dr. Anthony Magnetti

"Mechanisms of secretion in human airways." Invited Speaker, Institut Pasteur Euroconference: "Chronic Lung Diseases," Paris, France, June 2001.

"Growth Factors and Signal Transduction" Chair, Workshop organized by the National Institute of Environmental Health Sciences; Research Triangle Park, North Carolina, April, 2001

"Inflammatory mechanisms in airway epithelium." Invited Speaker, AstraZeneca Symposium on "COPD: Into the New Millennium." Lund, Sweden, April, 2001.

University of Pennsylvania School of Medicine, Philadelphia, PA. March, 2001. Host: Dr. Reynold Panetierri

Uniformed Services University of Health Sciences, Bethesda, MD. February, 2001. Host: Dr. Elliott Kagan

University of Alabama at Birmingham, Birmingham, AL, February, 2001. Host: Dr. Sadis Matalon.

**2000**

Harvard University School of Public Health, Boston, MA, November, 2000. Host: Dr. Lester Kobzik.

Vanderbilt University School of Medicine, Nashville, TN, October, 2000. Host: Dr. James Sheller.

AstraZeneca corporation, Stockholm, Sweden, September, 2000. Host: Dr. Karin Kristennson.

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"Molecular Regulation in Mucus Secretion." Invited Speaker, "Inflammatory Responses to Inhaled Agents;" British Association For Lung Research Summer 2000 meeting, Napier University, Edinburgh, Sept. 2000.

"Intracellular Signaling Cascades in Airway Epithelial Mucin Secretion". Invited Speaker, ATS-NIEHS Workshop "Lung Disease and the Environment--Where do We Go From Here"; ALA/ATS Annual Meeting, Toronto, Canada, May, 2000

"How Cells Secrete: From the Liver to the Lung." Third Annual Michael A. Gerber Memorial Lecture; Tulane University Medical Center; New Orleans, LA, April, 2000

Chemical Industry Institute of Toxicology, Research Triangle Park, NC, April, 2000. Host: Dr. Jeffrey Everitt

University of Maryland @ Baltimore, Baltimore, MD, March 2000. Host: Dr. K. Chul Kim.

**1999**

"Intracellular signaling mechanisms controlling airway mucin secretion" Invited Speaker and member of Organizing Committee/Session Chair, Third International Symposium on Mucus and Mucociliary Interactions, Sirmione, Italy, November, 1999.

National Institute of Environmental Health Sciences, Research Triangle Park, NC, October, 1999. Host: Dr. Peter Koo.

University of California at Davis, September, 1999. Host: Dr. Reen Wu

"Effects of Ozone on Isolated Biological Systems" Invited Speaker, World Congress on Ozonotherapy, Verona, Italy, March 1999.

Transatlantic Airway Conference, Key Biscayne, FL, January, 1999. Invited Discussant.

University of Miami School of Medicine, January, 1999. Host: Dr. Matthias Salathe.

**1998**

"Air-liquid interface culture systems for exposure of differentiated cells to oxidant stresses". Invited Speaker, 5th Annual Meeting of Oxygen Society, Washington, DC, November, 1998.

Schering-Plough Research Institute, Kenilworth, NJ. Host, Dr. Michael Minnicozzi, October 1998.

Johns Hopkins School of Hygiene and Public Health, Baltimore, MD. Host: Dr. Arthur Freed,

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September, 1998.

University of Paris, Jussieu, Paris, FR. Host: Dr. Frederic Tournier, July, 1998

University of Valencia, Valencia, Spain. Host, Dr. Jose Vina, July, 1998

Pfizer Corporation, Sandwich, UK. Host, Dr. Michael Mosley, July, 1998

University of Minnesota Medical College, Minneapolis, MN. Host, Dr. David Ingbar, June, 1998

Hoechst Marion Rousel, Inc. Bridgewater, NJ. Host, Dr. Martin Wasserman, June, 1998

"Signaling in Airway Epithelial Mucin Gene Expression and Secretion". Invited Speaker, Symposium on Airway Epithelium, ALA/ATS Annual Meeting, Chicago, IL, April, 1998.

University of Illinois @ Chicago, Chicago, IL. Host, Dr. J. Szjnader, April, 1998.

"Effects of Inflammatory Mediators and Drugs on Mucus Secretion and Mucociliary Function." Invited Speaker, EuroConference on "New Therapeutic Approaches for Allergic Diseases of the Respiratory Tract," Institut Pasteur, Paris, FR. April, 1998.

"Reactive Species in Signaling by Airway Epithelium" National Institute of Environmental Health Sciences, RTP,NC. Host, Dr. Ronald P. Mason, January, 1998.

"Pulmonary and Respiratory Cell Biology: normal function and defense" Glaxo-Wellcome, Inc., RTP, NC. Host: Dr. Paula Rogenes, January, 1998.

**1997**

Schering-Plough Research Institute, Kenilworth, NJ. Host, Dr. Michael Minnicozzi, February, 1997.

"Intracellular Regulation of Mucin Secretion". Invited Speaker, International Congress on Cilia, Mucus and Mucociliary Interactions, Jerusalem, Israel, February, 1997

University of Indiana, Indianapolis, Indiana, Host: Dr. Joe N. Garcia, March, 1997

USUHS, Bethesda, MD, Host, Dr. Elliott Kagan. March, 1997

University of Pittsburgh, Pittsburgh, PA, Host; Dr. Bruce Pitt, April 1997

Washington University, St. Louis, MO, Host: Dr. William C. Parks, May, 1997

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University of California at San Francisco, San Francisco, CA, Host: Dr. V. Courtney Broaddus, May, 1997.

"Epithelial and Interstitial Injury and Remodeling." Invited Speaker, American Society of Investigative Pathology Symposium, Experimental Biology '97, New Orleans, LA, April, 1997

"Airway epithelial cell differentiation at the air/liquid interface." Invited Speaker, American Thoracic Society/American Lung Association Postgraduate Course on Lung Cell and Tissue Culture, San Francisco, CA. May 1997

"Oxidant-regulated gene expression in inflammatory lung disease." Invited Speaker: NATO Advanced Study Institute: Acute Respiratory Distress Syndrome: Cellular and Molecular Mechanisms and Clinical Management". Corfu, Greece. June, 1997

Pfizer, Inc., Sandwich, England. Host: Dr. Michael Mosely, June, 1997

**CHAIR:** Second International Meeting on Oxygen and Nitrogen Radicals and Cellular Injury, Durham, NC, September, 1997.

Inspire Pharmaceuticals, Inc. Durham, NC: Host: Dr. Neil Jones, September, 1997

University of North Carolina, Chapel Hill, NC, Host: Dr. William Davis, October, 1997.

"Signal Transduction Mechanisms of Airway Mucin Secretion" Invited Speaker, Symposium "Conducting Airway Mucus in Health and Disease", Annual Meeting of The Formosan Medical Association,, Taipei, Taiwan. November, 1997.

"Signaling mechanisms associated with airway mucin secretion" Invited Speaker, Glaxo-Wellcome Inc., RTP, NC. November, 1997.

"New areas of epithelial cell research" Invited Speaker, Glaxo Wellcome, Inc., RTP, NC. December, 1997.

**1996**

"Signal transduction mechanisms associated with exposure of airway epithelial cells to ozone." Invited Speaker, Gordon Research Conference on Oxygen Radicals, Ventura, CA, February, 1996

"Studies on guinea pig airway mucin secretion." Pfizer Corporation, Mystic, CT., February, 1996, Host: Dr. Claudia Turner

"NO; A key signaling molecule in airway mucin secretion". Academia Sinica. Taiwan, ROC, April, 1996. Host: Dr. Te-Chang Lee



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"A common pathway for mediator-induced mucin secretion". Invited Speaker; 9th World Cong Bronchology & World Cong Bronchoesophagology, Taipei, Taiwan, ROC, April, 1996

"Role of epithelium in airway inflammation." Invited Speaker; Symposium on Bronchial Secretions, Seoul, ROK, April, 1996

"Nitric oxide as a key signaling molecule in airway mucin secretion". Department of Biochemistry, University of Nebraska Medical Center, Omaha, NE. April, 1996. Host: Dr. Pi-Wan Cheng

"Nitric oxide mediates airway epithelial mucin secretion". Invited Speaker, Symposium on Nitric Oxide, Schering Corporation, Kenilworth, NJ, May 1996

"Studies on airway mucin secretion." Department of Pulmonary Medicine, Sterling-Winthrop University Hospital, Mineola, NY., May 1996. Host: Dr. Stuart Horowitz

"Response of airway epithelium to oxidant stress: signal transduction mechanisms." Invited Speaker: American Thoracic Society/American Lung Association symposium on Oxidants as Second Messengers, New Orleans, LA, May 1996

"NO regulates gene expression of IL-6 in airway epithelial cells." Invited Speaker, Advanced NATO Workshop, Vascular Endothelium: Pharmacologic and Genetic Manipulations," Crete, Greece, June 1996

"Role of reactive oxygen and nitrogen species in the response of airway epithelium to particulates." Invited Speaker, Sixth International Meeting on the Toxicology of Natural and Man-Made Fibrous and Non-Fibrous Particles. Lake Placid, NY, September, 1996

"Studies utilizing air/liquid interface cultures of human bronchial epithelium." Environmental Protection Agency, Research Triangle Park, NC, October, 1996. Host: Dr. Robert Devlin

"Airway epithelium: a primary source for growth factors related to development of fibrosis?" Invited Speaker, Ninth International Colloquium on Pulmonary Fibrosis, Oaxaca, Mexico, November, 1996.

**1995**

"Interactions between oxidant gases and airway epithelium: in vitro - in vivo correlations". Invited Speaker and Member of Organizing Committee, Symposium: Correlations between in vitro and in vivo investigations in inhalation toxicology. Hannover Medical School, Hannover, Germany, February, 1995.

"Conducting airway epithelium as a source of mediators." Invited speaker, symposium/refreshers

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course for American Association of Anatomists, Atlanta, GA. April, 1995.

"Interactions of pollutants with epithelium: role of reactive oxygen and nitrogen species." Invited Speaker and Roger. S. Mitchell Lecturer, Aspen Lung Conference, Aspen, CO, June 1995.

"Mechanisms of mucin gene expression and secretion." Invited Speaker, Asthma: Theory to Treatment", Chicago, Il, July 1995.

"Mechanisms of ICAM-1 expression in airway epithelial cells." Invited Speaker, International Respiratory Forum, London, UK, November, 1995.

"Nitric Oxide regulates airway epithelial mucin secretion". Environmental Biology Center, Tulane University, New Orleans, LA, November 1995. Host: Dr. Arnold Brody.

"Nitric Oxide: A key signaling molecule in airway epithelium". Department of Anesthesiology, University of Alabama, Birmingham, AL, December 1995. Host: Dr. Sadis Matalon.

"Nitric oxide regulates airway mucin secretion". Department of Anatomy, University of California at Davis, Davis, CA, December, 1995. Host: Dr. Reen Wu.

## **PUBLICATIONS**

- Adler KB**, Wooten O, Philipoff W, Lerner E, Dulfano MJ: Physical properties of sputum. III. Rheological variability and intrinsic relationships. *Am Rev Resp Dis*, 106:86-96, 1972.
- Dulfano MJ, **Adler KB**, Wooten O: Physical properties of sputum. IV. The effects of 100% humidity and water mist. *Am Rev Resp Dis*, 107:130-133, 1973.
- Adler KB**, Wooten O, Dulfano MJ: Mammalian respiratory mucociliary clearance. *Arch Environ Health*, 27:364-369, 1973.
- Adler KB**, Dulfano MJ, Wooten O: Physical properties of sputum. V. The effects of time, freezing and thawing on viscoelasticity measurements. *Am Rev Resp Dis*, 109:490-492, 1974.
- Dulfano MJ, **Adler KB**: Physical properties of sputum. VII. Rheologic properties and mucociliary transport. *Am Rev Resp Dis*, 112:341-348, 1975.
- Adler KB**, Dulfano MJ: The rheological factor in mucociliary clearance. *J Lab Clin Med*, 88:22-30, 1976.
- Adler KB**, Fand I: The cilioinhibitory effect of phenothiazine in-vitro and its antagonism by calcium. *Arch Intern Exp Ther*, 227:309-322, 1977.
- Mossman BT, **Adler KB**, Craighead JE: The interaction of carbon particles with hamster tracheal epithelium in organ culture. *Environ Res*, 16:110-122, 1978.
- Adler KB**, Brody AR, Craighead JE: Association of microtubules and microfilaments with mucin granules in tracheal goblet cells-their possible role in exocytosis. *Proc 9th Internat Congress Electr Microsc*, 2:500-501, 1978.
- Davis GS, Brody AR, **Adler KB**: Functional and physiological correlates of human alveolar macrophage cell shape and surface morphology. *Chest*, 75S:280-282, 1979.
- Adler KB**, Davis GS, Woodworth CW, Brody AR: The human pulmonary alveolar macrophage: two distinct morphological populations. *Scan Electr Microsc*, 3:921-928, 1979.
- Mossman BT, Ezerman EB, **Adler KB**, Craighead JE: Isolation and spontaneous transformation of cloned lines of hamster tracheal epithelial cells. *Cancer Res*, 40:4403-4408, 1980.
- Mossman BT, **Adler KB**, Craighead JE: Cytotoxic and proliferative changes in tracheal organ and cell cultures after exposure to mineral dusts. In: "The In-Vitro Effects of Mineral Dusts" (Brown RC, Gormley IP, Chamberlain M, Davies R, eds.), Academic Press, London, pp. 241-250, 1980.

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Davis GS, Brody AR, **Adler KB**: Changes in the surface morphology of human alveolar macrophages induced by tobacco and marijuana smoking. *Exp Lung Res*, 1:281-293, 1980.

**Adler KB**, Brody AR, Craighead JE: Studies on the mechanism of mucin secretion by cells of the porcine tracheal epithelium. *Proc Soc Exp Biol Med*, 166:96-106, 1981.

**Adler KB**, Craighead JE, Vallyathan NV, Evans JN: Actin-containing cells in human pulmonary fibrosis. *Amer J Pathol*, 102:427-438, 1981.

Evans JN, **Adler KB**: The lung strip: Evaluation of a method to study contractility of pulmonary parenchyma. *Exp Lung Res*, 2:187-194, 1981.

**Adler KB**, Hardwick DH, Craighead JE: Effect of cholera toxin on secretion of mucin by explants of guinea pig trachea. *Lab Invest*, 45:372-377, 1981.

Evans JN, Kelley J, Low RB, **Adler KB**: Increased contractility of isolated lung parenchyma in an animal model of pulmonary fibrosis induced by bleomycin. *Am Rev Resp Dis*, 125:89-94, 1982.

**Adler KB**, Hardwick DH, Craighead JE: Porcine tracheal goblet cell ultrastructure: a three dimensional reconstruction. *Exp Lung Res*, 3:69-80, 1982.

**Adler KB**, Winn WC Jr., Hardwick DH, Craighead JE: Effects of bacterial toxins on secretion of mucin by rodent trachea in-vitro. *Chest*, 81:37S-39S, 1982.

Mossman BT, **Adler KB**, Jean L, Craighead JE: Mechanisms of hypersecretion in rodent tracheal explants after exposure to chrysotile asbestos: Studies using lectins. *Chest*, 81:23S-25S, 1982.

**Adler KB**, Winn WC Jr, Alberghini TV, Craighead JE: Stimulatory effect of *Pseudomonas aeruginosa* on mucin secretion by respiratory epithelium. *J Am Med Assoc*, 249:1615-1617, 1983.

Craighead JE, **Adler KB**, Butler GB, Emerson RJ, Mossman BT, Woodworth CW: Biology of Disease: Health effects of Mount St. Helens volcanic dust. *Lab Invest*, 48:5-12, 1983.

Brody AR, Hill L, Stirewalt W, **Adler KB**: Actin-containing microfilaments of pulmonary epithelial cells provide a mechanism for translocating inhaled asbestos to the interstitium. *Chest* 83:11S-13S, 1983.

Evans JN, Low RB, Kelley J, Krill J, **Adler KB**: The myofibroblast in pulmonary fibrosis. *Chest* 83:97S-99S, 1983.

**Adler KB**, Alberghini TV, Counts DF, Auletta F: Cellular mechanisms of mucus secretion by rabbit and

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human cervical explants in-vitro. Biol Reprod 29:751-765, 1983.

Low RB, Mitchell JW, Evans JN, **Adler KB**: Contractile proteins of the lung. Current Probs Clin Biochem 13:149-156, 1983.

Evans JN, Krill J, **Adler KB**, Low RB, Kelley J: Active cellular control of alveolar compliance. Current Probs Clin Biochem 13:142-148, 1983.

**Adler KB**, Krill J, Alberghini T, Evans JN: Effects of cytochalasin D on smooth muscle contraction. Cell Motility 3:545-551, 1983.

Low RB, Mitchell JW, Evans JN, **Adler KB**: Actin content in normal and bleomycin fibrotic rat lung. Am Rev Resp Dis 129:311-316, 1984.

Woodcock-Mitchell J, **Adler KB**, Low RB: Immunohistochemical identification of cell types in normal and in bleomycin-induced fibrotic rat lung. Cellular origins of interstitial cells. Am Rev Resp Dis 130:910-916, 1984.

**Adler KB**, Mossman BT, Butler GB, Jean LM, Craighead JE: Interaction of Mount St. Helens' volcanic ash with cells of the respiratory epithelium. Environ Res 35:346-361, 1984.

**Adler KB**, Butler GB, Hemenway DR, Schwarz JE, Banks PO, Evans JN: Exposure of small airways to cristobalite in-vitro. In: Beck E, Bignon J (eds.): In-vitro Effects of Mineral Dusts. NATO ASI series, Vol G-3, Springer Verlag, Heidelberg, FRG, pp. 293-301, 1985.

Callahan LM, Evans JN, **Adler KB**: Alterations in the cellular population of the alveolar wall in an animal model of fibrosis: a morphometric study. Chest, 89:189S-190S, 1986.

Brody AR, Hill LH, Hesterberg TW, Barrett JC, **Adler KB**: Intracellular translocation of inorganic particles. In: Clarkson TW, Sager PR, Syversen(eds.): The Cytoskeleton. Plenum Publishing Corp, pp.221-227, 1986.

**Adler KB**, Callahan LM, Evans JN: Alterations in the cellular population of the alveolar wall in bleomycin-induced pulmonary fibrosis in rats: An ultrastructural morphometric study. Am Rev Resp Dis 133:1043-1048, 1986.

**Adler KB**: "Mucin secretion by respiratory tract tissue in vitro." In: Schiff LJ (ed.): In Vitro Models of Respiratory Epithelium", CRC Press, Boca Raton, FL, pp. 27-50, 1986.

**Adler KB**, Schwarz JE, Repine JE: Oxygen free radicals stimulate secretion of mucin by rodent respiratory epithelium in-vitro. In: Romig AD Jr., Chambers WF (eds.): Microbeam Analysis - 1986. San Francisco Press, Inc., San Francisco, CA, pp.571-573, 1986.

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**Adler KB**, Hendley DD, Davis GS: Bacteria associated with chronic obstructive pulmonary disease elaborate extracellular products that stimulate secretion of mucin by explants of guinea pig airways. *Am J Pathol* 125:501-514, 1986.

**Adler KB**, Schwarz JE, Anderson WH, Welton AF: Platelet-activating factor stimulates secretion of mucin by explants of rodent airways in organ culture. *Exp Lung Res* 13:25-43, 1987.

Butler GB, **Adler KB**, Evans JN, Szarek J: Modulation of rabbit airway smooth muscle responsiveness by respiratory epithelium: involvement of an inhibitory metabolite of arachidonic acid. *Am Rev Resp Dis* 135:1099-1104, 1987.

**Adler KB**, Schwarz JE, Whitcutt JM, Wu R: A new chamber system for maintaining differentiated guinea pig respiratory epithelial cells between air and liquid phases. *Biotechniques* 5:462-467, 1987.

Coflesky JT, **Adler KB**, Evans JN: Alterations in pulmonary vascular responsiveness following hyperoxic injury to the lung. *Chest* 93:147S-149S, 1988.

Whitcutt JM, **Adler KB**, Wu R: A biphasic chamber system for maintaining polarity of differentiation of cultured respiratory tract epithelial cells. *In Vitro Cell Develop Biol* 24:420-428, 1988.

Coflesky JT, **Adler KB**, Woodcock-Mitchell J, Mitchell J, Evans JN: Proliferative changes in the pulmonary arterial wall during short-term hyperoxic injury to the lung. *Am J Pathol* 132:563-573, 1988.

**Adler KB**, Akley NJ: Oxygen radicals stimulate secretion of mucin by rodent airway epithelial cells in organotypic culture. In: Cerrutti P, Fridovich I, McCord J (Eds): "Oxy-radicals in molecular biology and pathology", Alan R. Liss, Inc., New York, NY, 1988, pp.101-108.

Low RB, **Adler KB**, Woodcock-Mitchell J, Giancola MS, Vacek PM: Bronchoalveolar lavage lipids during development of bleomycin-induced fibrosis in rats: relationship to altered epithelial cell morphology. *Am Rev Resp Dis* 138:709-713, 1988.

Brody AR, Bitterman PB, **Adler KB**, Rannels DE, Thet LA, Rom WN, Rennard SI: The lung matrix and inflammation: Part II. Biochemical and molecular mechanisms of fibrogenesis: implications for environmental lung disease. *Am Rev Resp Dis* 138:1056-1057, 1988.

**Adler KB**, Low RB, Leslie KO, Mitchell J, Evans JN: Biology of disease: Contractile cells in normal and fibrotic lungs. *Lab Invest* 60:473-485, 1989.

Mitchell J, Woodcock-Mitchell J, Reynolds S, Low R, Leslie K, **Adler KB**, Gabbiani G, Skalli O: Alpha smooth muscle actin in parenchymal cells of bleomycin-injured rat lung. *Lab Invest* 60:643-650,

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1989.

**Adler KB**, Akley NJ, Ayers G, Holden-Stauffer WJ, Khosla JB, Kim KC: A novel culture system for maintaining differentiated epithelial cells between air and liquid phases: A possible model for studying interactions between mineral fibers, gases and airway epithelium. In: Wehner, AP (Ed.); Biological Interaction of Inhaled Mineral Fibers and Cigarette Smoke, Proceedings of an International Symposium/Workshop, Batelle Press, Columbus, OH, 1989. pp. 381-390.

**Adler KB**, Cheng PW, Kim KC: Characterization of guinea pig tracheal epithelial cells maintained in biphasic organotypic culture: cellular composition and biochemical analysis of released glycoconjugates. *Am J Resp Cell Molec Biol* 2:145-154, 1990.

**Adler KB**, Holden-Stauffer WJ, Repine JE: Oxygen radicals stimulate release of high molecular weight glycoconjugates by cell and organ cultures of rodent respiratory epithelium via an arachidonic acid-dependent mechanism. *J Clin Invest* 85:75-85, 1990.

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PATENT

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## Exhibit 2

# Mucous-Cell Metaplasia and Inflammatory-Cell Recruitment Are Dissociated in Allergic Mice after Antibody- and Drug-Dependent Cell Depletion in a Murine Model of Asthma

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Inflammatory-cell infiltration and epithelial modifications are prominent lesions of the bronchial mucosa in asthma and in experimental allergic bronchopulmonary inflammation. However, the recruitment of inflammatory cells and their relationship to the epithelial modifications and to functional alterations such as bronchopulmonary hyperreactivity (BHR) are less known. We studied the mechanisms of antigen-dependent inflammatory-cell recruitment to the lungs and the associated lesions and their relationship using drug- and antibody-dependent cell-depletion procedures. A single intranasal ovalbumin challenge in BP2 mice was found to induce hyperreactivity within 1 h after challenge, followed by the massive infiltration of immunoglobulin (Ig)E-bearing polymorphonuclear leukocytes (PMN), and eosinophils, and by a mucous-cell metaplasia of the bronchiolar epithelium. Similarly challenged BALB/c mice did not exhibit BHR, despite a moderate recruitment of inflammatory cells and mucous-cell metaplasia. Inflammatory-cell recruitment, mucous-cell metaplasia, and BHR were prevented by prior antibody-dependent depletion of CD3<sup>+</sup> lymphocytes and partially inhibited by the depletion of CD4<sup>+</sup> lymphocytes. Treatment with the granulocytopenic drug vinblastine before challenge completely abolished the recruitment of granulocytes without affecting the antigen-induced mucous-cell metaplasia. In this study two new key elements of the murine model of allergic pulmonary inflammation are described: the recruitment of IgE-bearing PMN between 3 and 72 h after challenge, and the dissociation between granulocytes and mucous-cell metaplasia. Haile, S., J. Lefort, D. Joseph, P. Gounon, M. Huerre, and B. B. Vargaftig. 1999. Mucous-cell metaplasia and inflammatory-cell recruitment are dissociated in allergic mice after antibody- and drug-dependent cell depletion in a murine model of asthma. *Am. J. Respir. Cell Mol. Biol.* 20:891–902.

The accumulation of inflammatory cells in the bronchial tissue during allergic reactions is T lymphocyte-dependent (1). Specifically, T cells from the Th2 subtype secrete cytokines that induce the maturation, migration, and accumulation of effector cells, particularly eosinophils. Ovalbumin (OVA)-immunized mice challenged locally with OVA respond with an influx of eosinophils, lymphocytes, and, to a lesser extent, other inflammatory cells into their airways (2, 3). These mice may display antigen-dependent functional respiratory disturbances similar to those of asthma, such as bronchopulmonary hyperreactivity (BHR).

Nevertheless, most of the protocols so far described in mice require multiple immunizations and/or challenges with antigen in order to achieve significant BHR (2, 4). Although multiple provocations during long periods of time may simulate the events of asthma, the mechanisms of inflammatory-cell recruitment are probably better understood under a single antigenic challenge. For this reason, we investigated inflammatory responses and BHR following a single antigenic challenge in the hyperimmunoglobulin (Ig)E BP2 mice. In previous studies, these mice were found to express BHR after four antigenic challenges (5) under conditions that left BALB/c mice normoresponsive. In the presently described single OVA challenge protocol, nonanesthetized BP2 mice became hyperreactive to inhaled methacholine. We followed the kinetics of recruitment of lymphocytes, eosinophils, mast cells, and non-mast cell IgE-bearing cells to the lungs, as well as the major epithelial modifications in the bronchioles, under both light and electron microscopy in BP2 and BALB/c mice. To study the role of inflammatory cells for BHR, we depleted T cells with antibodies and took advantage of the granulocytopenic effects of vinblastine (6, 7) to correlate granulo-

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**Abbreviations:** bronchoalveolar lavage fluid, BALF; bronchopulmonary hyperreactivity, BHR; eosinophil peroxidase, EPO; immunoglobulin, Ig; interleukin, IL; ovalbumin, OVA; periodic acid-Schiff and Alcian blue, PAS-Ab; enhanced pause, Penh; polymorphonuclear leukocytes, PMN.

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cyte (eosinophil) recruitment and epithelial modifications. Our results show that the recruitment of eosinophils and lymphocytes into the peribronchiolar and perivascular tissues within hours after a single antigen instillation into the murine airways is accompanied by that of IgE-bearing polymorphonuclear leukocytes (PMN), most likely basophils, and by a BHR that starts by 1 h after challenge. Epithelial cells of the bronchi and bronchioles undergo a mucous-cell metaplasia that starts within the first 6 h and persists for more than 11 d after challenge. Altogether, inflammatory-cell infiltration, mucous-cell metaplasia, and BHR were suppressed when CD3<sup>+</sup> lymphocytes were depleted, and were partially inhibited by anti-CD4 antibodies. Finally, vinblastine abrogated granulocyte recruitment into the lungs, but lymphocyte numbers and mucous-cell metaplasia remained unaffected. Mucus secretion is thus independent of the presence of granulocytes, but correlates with the presence of lymphocytes.

## Materials and Methods

### Animals, Immunizations, and OVA-Challenge Protocols

BP2 and BALB/c mice aged 6 to 8 wk (Centre d'Elevage R. Janvier, Le Genest Saint-Isle, France) were immunized subcutaneously at Day 0 and Day 7 with 100 µg OVA (Immunobiologicals, Lisle, IL) in the presence of 1.6 mg alum (Merck, Darmstadt, Germany) and diluted in a 0.4 ml saline solution. Intranasal OVA challenge was performed 1 wk after the second immunization, namely, at Day 14, with 10 µg OVA diluted in 50 µl of alum-free saline solution or with saline solution as a control under ether anesthesia. OVA challenge was performed once in one group and four times (twice a day) in another group. Six to 12 mice were taken at 1, 3, 6, 24, 48, and 72 h, and also at 6 and 11 d for the evaluation of pulmonary functions, and for histologic examination of the lungs. For electron microscopy studies of cells from the bronchoalveolar lavage fluid (BALF), mice were challenged four times to yield an optimum number of eosinophils (5).

### Evaluation of BHR

Unrestrained, conscious mice were placed in a plethysmographic chamber (Buxco Electronics, Sharon, CT), and respiratory parameters were measured before and after an aerosol of methacholine (Sigma-Aldrich, Stenheim, Germany) delivered for 20 s at  $3 \times 10^{-2}$  M in the aerosolator. The resistance was expressed as enhanced pause (Penh), calculated as:  $\{(\text{Expiratory time})/40\% \text{ of } (\text{Relaxation time}) - 1\} \times \{(\text{Peak expiratory flow})/(\text{Peak inspiratory flow})\} \times 0.67$ , according to the manufacturer's recommendations. Every 20 s an average value of Penh was recorded. For the graphic representation, each value was expressed for every minute. Accordingly, each point represents the average of three values.

### Sampling for Cytology and Histology

Mice were deeply anesthetized with urethane (intraperitoneally, 15 mg/10 g body wt), and the abdominal cavity was opened. Blood samples for serum were taken when needed; otherwise, animals were bled and killed by cutting of the large abdominal vessels. BALF was collected

by cannulating the trachea and washing the lung with a total of 4 ml saline solution ( $8 \times 0.5$  ml each). After cell counting, cytospin was prepared with a standard apparatus. Lungs were then inflated with 1 ml of 50% Optimum Cutting Temperature compound (Sakura Finetek, Torrance, CA) solution through the cannulated trachea. The left lobe was placed in a tube and immediately frozen in liquid nitrogen, and the remaining right lobes were fixed in 10% formaldehyde.

Formaldehyde-fixed samples were embedded in paraffin for standard histologic examination. Longitudinal and transversal sections of the major intrapulmonary bronchioles of 4-µm thickness were prepared and stained with hematoxylin and eosin and with periodic acid-Schiff and Alcian blue (PAS.Ab) reaction to study mucus-containing cells. PAS stains neutral mucin, and Ab stains acidic mucin. The thicknesses of the bronchial epithelium and the smooth muscle of the transversely sectioned preparations were measured using a computerized image analyzer with Optimas software (Bioscan, Inc., Edmonds, WA). In addition to the lung, secondary lymphoid organs, spleen, and mesenteric lymph nodes were also examined histologically when needed.

Uniform 6-µm cryostat sections containing longitudinal sections of the major bronchiole were prepared on Superfrost-plus slides (Menzel-Glasser, Braunschweig, Germany), rolled on cellophane paper, and stored frozen at  $-20^{\circ}\text{C}$  until further use. Slides with cytospin preparations were fixed and stored in the same manner.

### Histochemical Staining

Eosinophils were selectively stained for eosinophil peroxidase (EPO) on cryostat sections as described (8). Briefly, acetone-fixed sections were incubated for 10 min with a phosphate buffer, pH 7.4, containing diaminobenzidine 10 mg/13.3 ml, sodium cyanide, and hydrogen peroxide; washed; counterstained with Harris hematoxylin; and mounted with aquamount (Gurr; BDH Laboratories, Poole, UK). All other chemicals were from Sigma-Aldrich. Mast cells were stained and identified using a histochemical chloroacetate esterase stain for nonspecific esterase (9). Briefly, acetone-fixed sections were incubated with a solution containing the substrate naphthol AS-D acetate (5 mg dissolved in 0.5 ml dimethylformamide), 25 ml distilled water, 25 ml Tris buffer (pH 7.1), and 30 mg of Fast blue RR salt (all from Sigma-Aldrich).

### Immunohistochemistry

CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes and IgE-bearing cells were identified using immunohistochemical techniques. Anti-CD4 and anti-CD8 antibodies were purchased from Caltag (San Francisco, CA). Anti-IgE antibody EM95-3 (10) was kindly provided by Dr. Y. Chvatchko (Serono Pharmaceutical Research Institute, Geneva, Switzerland). Briefly, after covering the nonspecific site with 10% rabbit serum, sections were incubated for 1 h with the first antibody, a rat antimouse antibody (all antibodies were of rat origin), followed by washing in Tris-saline buffer (pH 7.6) and then incubation for 45 min with a second biotinylated rabbit antirat antibody (Dako A/S, Glostrup, Denmark). Washing was followed by another 45-min incubation with

alkaline phosphatase-labeled streptavidin (Dako A/S), followed by another washing. The reaction was revealed with an alkaline phosphatase substrate solution containing naphthol AS-MX phosphate, Fast red, and levamisole in Tris buffer (pH 8.2). Unless otherwise stated, all chemicals were from Sigma-Aldrich. Double immunohistochemical staining for IgE and for B lymphocytes or for neutrophils was performed consecutively using the same method. This was done to make a distinction between IgE-bearing PMN, neutrophils, and IgE-secreting and/or -bearing B lymphocytes. Antineutrophil and anti-B lymphocyte B220 antibodies were kindly provided by Dr. Geneviève Milon (Unité d'Immunophysiologie Cellulaire, Institut Pasteur, Paris, France).

### Electron Microscopy

Lungs from anesthetized mice were perfused first with phosphate-buffered saline (PBS) solution to remove blood, followed by a fixative of 1% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 (11). Lungs were slowly instilled intratracheally with the same fixative and then removed. The major intrapulmonary bronchiole was dissected out into four cubes of 1 mm each for each mouse and was left in a fixative containing 2.5% glutaraldehyde overnight. The next day, tissues were washed, postfixed in 2% osmium tetroxide, and processed as described (11).

In another group of mice, BALF and blood eosinophils from OVA-challenged mice were isolated by Percoll centrifugation as described (12). Because saline-challenged mice did not contain sufficient eosinophil numbers to serve as controls, blood eosinophils were collected from transgenic mice for interleukin (IL)-5, which do not become hyperreactive after a single antigenic provocation (13). Eosinophils were resuspended in PBS solution, washed, recentrifuged, and fixed in glutaraldehyde at 2.5% for 1 h at 4°C. After rinsing in phosphate buffer, they were centrifuged and resuspended with two drops of 8% bovine serum albumin and agglutinated by a brief incubation in two drops of 25% glutaraldehyde as described (11). The pellets were washed and postfixed in 2% osmium tetroxide for 1 h. They were then processed as described for fixed tissues (11).

### Th2 Cytokine Measurements

IL-4 and IL-5 were evaluated in BALF and in serum at different times after challenge. IL-4 was measured with an enzyme-linked immunosorbent assay technique (Valbio-tech, Paris, France). Briefly, 96-well plates were coated with 2 µg/ml of rat antimouse IL-4 (BVD4-1D11; Pharmingen, San Diego, CA), to which were added dilutions of recombinant (rc)IL-4 standard (7 to 1,000 pg/ml) or of the sample, followed by a biotinylated rat anti-IL-4 antibody (BVD6-24G2; Pharmingen) at 0.5 mg/ml. The reaction was revealed using a substrate containing *o*-phenylenediamine dihydrochloride in phosphate buffer and read at 490 nm.

IL-5 was measured using the immunometric assay as described (5). Briefly, 96-well plates were coated with 10 µg/ml of rat antimouse IL-5 (TRFK-4) to which were added an rcIL-5 standard (7.8 to 1,000 pg/ml) or the sample, followed by an AchE-labeled rat antimouse IL-5 antibody (TRFK-5) at 10 Ellman U/ml. Absorbance was read

at 405 nm. The lower limit of detection of both assays was approximately 5 pg/ml of the sample. Both TRFK-4 and TRFK-5 were purified from ascitic fluids (cloned hybridomas provided by Dr. P. Minoprio, Institut Pasteur).

### *In Vivo* Treatments

Hamster antimouse CD3 monoclonal antibody (mAb) 145-2C11 (14) (kindly provided by Dr. L. Majlessi, Unité d'Immunophysiologie Moléculaire, Institut Pasteur) or hamster IgG as a control was given once intravenously at a dose of 200 µg per animal. The next day mice were challenged intranasally with OVA. In another group of animals, OVA challenge was performed 1 wk after the treatment with anti-CD3. Administration of whole anti-CD3 antibody causes high morbidity and mortality from immunodepression and from excessive secretion of IL-2 and interferon-γ in mice (15). For this reason our mice were kept in rigid isolation in specific pathogen-free conditions to prevent secondary infection.

To deplete CD4 lymphocytes, rat antimouse CD4 mAb GK 1.5 (kindly provided by Dr. G. Bordenave, Unité d'Immunophysiologie Moléculaire, Institut Pasteur) or rat IgG as a control was given at a dose of 300 µg/mouse/d during 3 consecutive days, a week before challenge, insuring a maximum depletion of CD4 cells. In a preliminary test, one injection of 1 mg of anti-CD4 antibody failed to deplete lymphocytes and to inhibit eosinophil infiltration in BP2 mice, but depleted CD4 lymphocytes and inhibited eosinophil infiltration into the lungs in BALB/c mice (data not shown). We did not identify the cause of the relative resistance of BP2 mice to anti-CD4-dependent depletion. Depletion or absence of depletion of lymphocytes was verified in the spleen and mesenteric lymph nodes by fluorescence-activated cell sorter (FACS) analysis using labeled antibodies as described (16), and by histologic examination of the lymphoid organs.

To study whether granulocyte depletion might affect inflammatory-cell recruitment to the lungs and mucus production by bronchial epithelial cells and BHR, vinblastine (Lilly France, Saint Cloud, France) was administered intravenously at 5 mg/kg 72 h before the antigenic challenge. Because mucus production by epithelial cells is best quantified histologically 72 h after antigenic provocation, evaluation was performed at this time point.

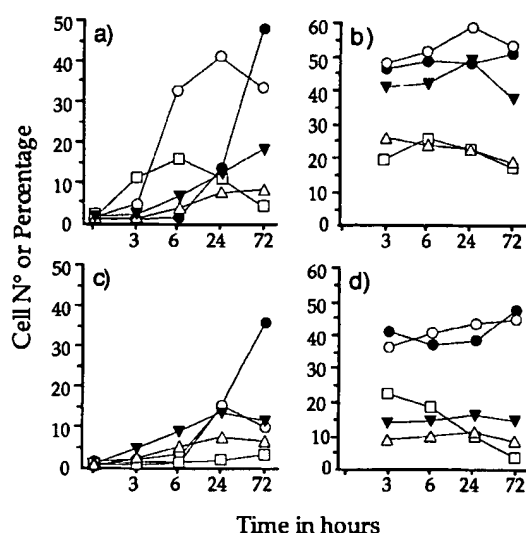
### Statistics

All results are presented as means ± SEM. Significance levels were calculated using the nonparametric Mann-Whitney U test, and  $P < 0.05$  was taken as significant difference between data (17).

### Results

#### Eosinophil and Lymphocyte Recruitment Is More Intense in BP2 Than in BALB/c Mice after a Single OVA Challenge

Saline-challenged control mice did not show eosinophil recruitment in the airways. In OVA-challenged mice, the kinetics of eosinophil recruitment to the airways were similar in BP2 and BALB/c mice, but the intensity differed: BP2 mice recruited > 2-fold more cells (measured at 24 h



**Figure 1.** Time-dependent inflammatory cell recruitment and epithelial mucus secretion in BP2 (a and b) and BALB/c (c and d) mice after one (a and c) or four (b and d) intranasal OVA challenges. Mice challenged with saline as antigen control did not show inflammatory cell infiltration or mucus secretion. Eosinophils (open circles) and CD4<sup>+</sup> (closed triangles) and CD8<sup>+</sup> (open triangles) lymphocytes were counted as cells per millimeter length in the major intrapulmonary bronchi. IgE<sup>+</sup> PMN (open squares) were counted as cells per square millimeter of the lung section. Mucus secretion (closed circles) was evaluated as the proportion of mucus-containing cells over total epithelial cells of the major intrapulmonary bronchi. Standard errors (not shown for simplicity) were below 12%.

after challenge) than BALB/c mice (Figures 1a, 1c, 2a, and 2b). These differences were particularly clear after a single provocation, but persisted after four challenges (Figures 1b and 1d).

Three hours after challenge, eosinophils margined onto the endothelial surface. By 6 h, they started to infiltrate the peribronchial and perivascular connective tissues. Their numbers increased by 24 h, to peak by 48 to 72 h (Figure 1a), a time when focal eosinophilic alveolitis was also seen. Peribronchial and perivascular infiltration increased and alveolar eosinophilia decreased with time. Tissue eosinophilia decreased slightly but still persisted at Day 11 (not shown). Four OVA challenges increased the numbers of eosinophils and T lymphocytes infiltrating the lung and prolonged the persistence of IgE-bearing PMN numbers. As observed using the EPO stain, no extracellular eosinophil granules were present at any time.

Saline-challenged control mice did not show lymphocyte infiltration in the airways. T lymphocytes infiltrated the peribronchial and perivascular tissues as early as 6 h after challenge and their numbers increased with time (Figure 1a). At 24 h and onward, B lymphocytes were the most numerous cells recruited, followed by CD4<sup>+</sup> T lymphocytes (not shown). Lymphoid follicles containing germinal centers were clearly noted from 6 h on after challenge, particularly in BP2 mice. The intensity of lymphocyte recruitment increased when mice were challenged four times.

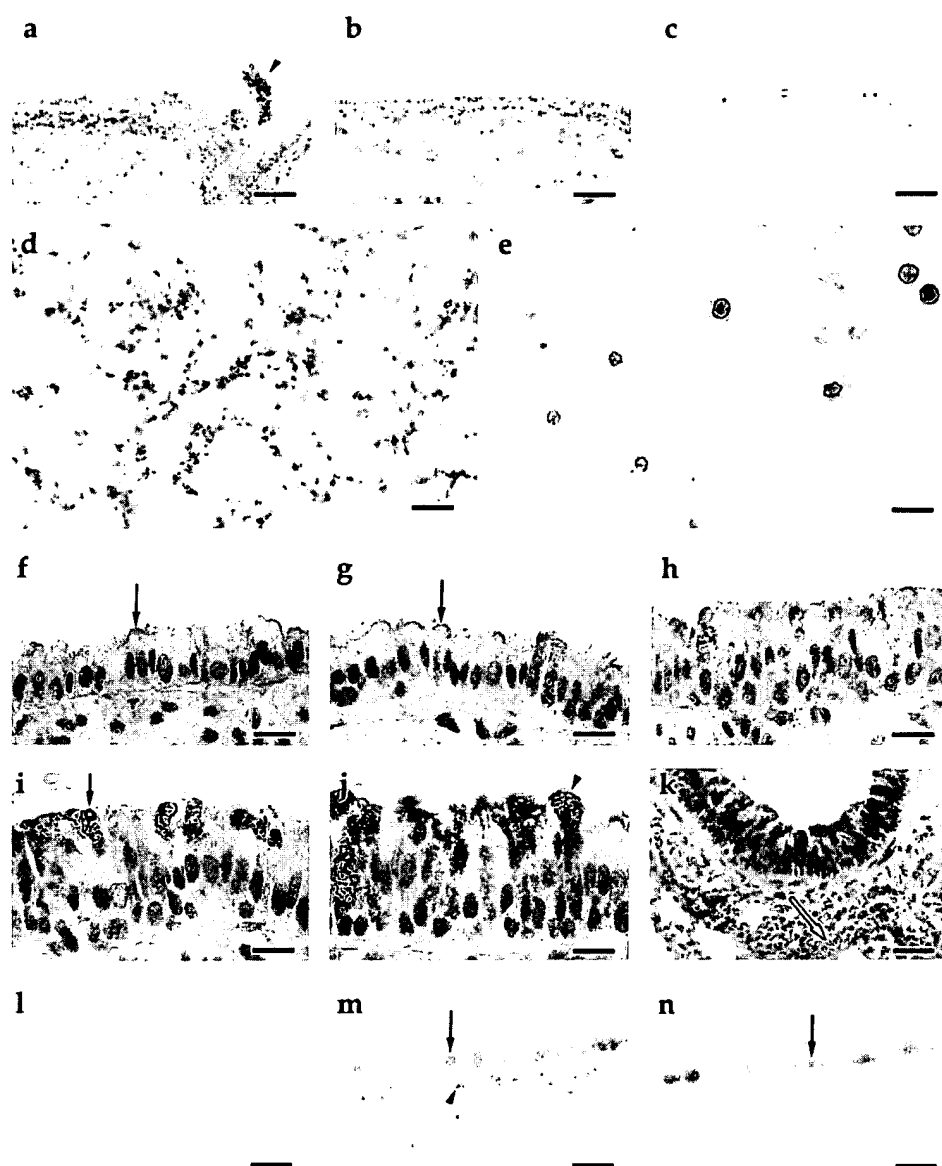
In electron microscopic preparations, numerous eosinophils were observed in the submucosa of the airways. Nevertheless, in both strains of mice no eosinophils with morphologic aspect of activation were seen, and neither rarefaction of the crystalloid or of the matrix of the granules (a morphologic counterpart of activation) nor free granules were observed in the submucosa or in the epithelium (not shown). Eosinophils freshly isolated from the BALF and from blood did not show altered granules or other modifications, such as the increase in the number of lipid bodies described for human eosinophils from allergic lesions (Figure 3c). In one sample from an OVA-challenged BP2 mouse, free eosinophils were noted in the bronchial lumen next to the surface of the epithelium, displaying characteristics of degeneration such as swollen and disrupted mitochondria and disintegrating granules coming out of the cell (Figure 3d).

#### IgE-Bearing PMN Are Recruited into the Lungs within the First Hours after OVA Challenge

In immunized saline-challenged controls, faint IgE staining was observed only on a few mast cells and on lymphocytes, and very strong staining was noted on PMN circulating in the capillaries. In OVA-challenged mice, the intensity of mast-cell staining and numbers of IgE-bearing PMN increased (Figure 2d), whereas lymphocytes were still very faintly stained, even though their number increased. The first cells to infiltrate the lungs 3 to 6 h after challenge were IgE-bearing PMN. From double immunohistochemical or combined immunohistochemical and histochemical stainings, these IgE-bearing PMN were negative for EPO, for the antineutrophil antibody NIMP-R14, for the B-lymphocyte marker B220 (Figure 2e), and for nonspecific esterase (18), as seen both in histologic preparations and in cytospin preparations of BALF. This, and the morphology of the cells in the cytospin (Figure 2e), allowed us to conclude that these IgE-bearing PMN are basophils. The kinetics of infiltration of these IgE-bearing PMN after a single OVA challenge are presented in Figure 1. The absence of a significant increase in the recruitment of IgE-bearing PMN after a single OVA challenge in BALB/c mice, which are not turned hyperreactive using the single antigenic provocation protocol, is noteworthy. The number of IgE-bearing PMN decreased rapidly to basal values by 72 h in BP2 mice challenged once (Figure 1a), but after four OVA challenges persisted for longer (Figure 1b).

#### No Variation in Mast Cell Numbers after OVA Challenge in Mice

Mast cells were scarce and localized on the first 2 to 3 mm of the longitudinally sectioned major intrapulmonary bronchioles of mice (Figure 2c). Slightly more mast cells were seen in the lungs of BALB/c than in those of BP2 mice ( $5.1 \pm 3.8$ /section of the left lobe for BP2, and  $6.5 \pm 3.5$  for BALB/c,  $P < 0.02$ ). In general, mast cells were more concentrated in the connective tissue between the large vessels and the major bronchiole at the level of the hilus of each lobe, and were rarely seen deep in the lung parenchyma. No significant differences in mast cell numbers were noted between OVA-challenged mice and their respective saline-challenged control mice.



**Figure 2.** Characterization of inflammatory cells and epithelial changes in the airways of OVA-challenged mice. (a and b) OVA-induced recruitment of eosinophils (brown) into the bronchiolar wall following a single OVA challenge is more intense in BP2 (a) than in BALB/c (b) mice. Note the presence of the cluster of eosinophils in the bronchiolar lumen in (a) (arrowhead). Histochemical staining for EPO on frozen lung sections was revealed with diaminobenzidine substrate. Bar = 160  $\mu$ m. (c) Localization and distribution of mast cells (blue-stained cells) in the mouse lung is limited to the first 2 to 3 mm of the major intrapulmonary bronchiole. Staining of non-specific esterase for proteases on frozen lung section was revealed with fast blue substrate. Bar = 160  $\mu$ m. (d) OVA-induced recruitment of IgE-positive PMN (red-stained cells). Slides of frozen lung sections were stained with the indirect labeled-antibody staining method, revealed with fast red and counterstained with hematoxylin. Bar = 30  $\mu$ m. (e) IgE-positive PMN (red-stained cells) in cytopsin preparation of the BALF from OVA-challenged BP2 mice. To show that these cells are not B lymphocytes, double staining for B220, a B lymphocyte marker (blue-stained cells), was performed. Slides were stained with the indirect labeled-antibody staining method, revealed with fast red (IgE) and fast blue (B220), and counterstained lightly with Metanil Yellow. Bar = 30  $\mu$ m. (f to k) Time-

dependent changes in bronchiolar epithelium of mice after a single OVA challenge, showing a saline-challenged control (f), and 6 (g), 24 (h), 48 (i), and 72 h (j and k) after OVA challenge. PAS-positive mucus granules developed in the apical pole of the cytoplasm (arrow) and increased with time. Mucus was also excreted to the lumen (arrowhead in j). White arrows in (k) show eosinophils infiltrating the submucosa. PAS reaction (purple) counterstained with hematoxylin. Bars: f to j, 10  $\mu$ m; k, 40  $\mu$ m. (l to n) Vinblastine abrogates eosinophil (arrowhead) recruitment into the lung but not mucous-cell metaplasia (arrow). (l) Saline-treated and saline-challenged control mice. (m) Saline-treated and OVA-challenged mice with eosinophil infiltrating the bronchiolar wall (arrowhead). (n) Vinblastine-treated and OVA-challenged mice. Combined EPO (red-brown) and PAS.Ab reaction (blue) on frozen lung sections without counterstaining. EPO was revealed with diaminobenzidine substrate. Bar = 160  $\mu$ m.

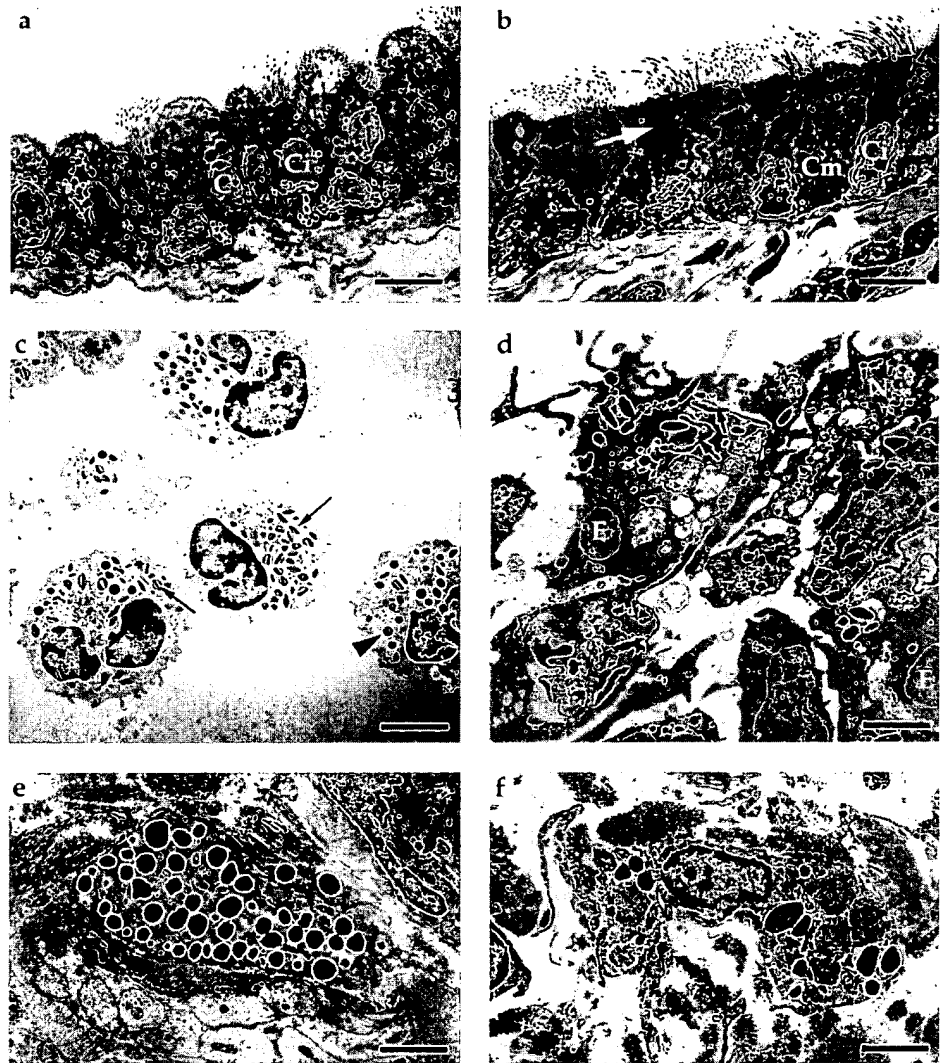
Only a few mast cells, with piecemeal degranulation, were seen in the electron microscopic preparations of bronchial wall in both BP2 and BALB/c mice challenged with OVA (Figure 3f).

#### Epithelial Lesions after OVA Challenge Consist Essentially of Mucous-Cell Metaplasia

OVA-challenged BP2 and BALB/c mice showed a significant increase in the height of the bronchial epithelial lining

and in the thickness of the smooth muscle at 72 h after challenge (Figure 4), when compared with saline-challenged controls. The other major epithelial change observed was the appearance of PAS.Ab-positive mucus-secreting cells in the epithelium. Approximately 50% of the cells of the major bronchioles of mice are Clara cells, very few of which showed PAS.Ab-positive secretory granules in the apical part of the cytoplasm. In OVA-challenged mice, secretory granules formed by both acidic (Alcian

**Figure 3.** Electron micrographs of tissues and of cells isolated from the BALF of OVA-challenged mice and their controls. (a) Bronchiolar epithelium of saline-challenged control mouse showing alternating ciliated cells (Ci) and nonciliated or Clara cells (C). Bar = 6  $\mu$ m. (b) Bronchiolar epithelium from a mouse 48 h after OVA challenge showing alternating ciliated (Ci) and nonciliated (C) cells containing mucus granules (white arrow) in their cytoplasm. Bar = 6  $\mu$ m. (c) Percoll-isolated eosinophils from the BALF of OVA-challenged BP2 mice show a normal structure. Mature granules (arrow) and immature granules (arrowhead) are shown. Note the absence of piecemeal degranulation and lipid bodies. Bar = 2  $\mu$ m. (d) Morphology of eosinophils *in situ* in the bronchiolar lumen. Degenerating eosinophils (E) in the bronchiolar lumen with disintegrating granules (arrows), mitochondrial ballooning, and a neutrophil (N). This was prepared from a cluster of eosinophils such as those observed in the bronchiolar lumen (Figure 2a, arrowhead). Bar = 3.5  $\mu$ m. (e) Mast cell from saline-challenged control mouse with dense and normally appearing granules. Bar = 2  $\mu$ m. (f) Mast cell from OVA-challenged mouse with rarefied granules, a morphologic aspect of degranulation. Bar = 2  $\mu$ m.



blue-stainable) and neutral (PAS-stainable) mucus started to appear in some Clara cells by 6 h. At 24 h, the number of granules increased substantially, and by 48 to 72 h after challenge almost all Clara cells (i.e., 50% of the total epithelial cell numbers) were transformed into mucus-secreting cells (Figures 2f to 2k). This was confirmed in electron microscopic preparations of the bronchiolar epithelium (Figure 3b). When individual cells were analyzed, the mucus granules started to appear in the apical pole of the cells and gradually increased in numbers in the mid- and basal parts. Mucus was also secreted in the bronchial lumen (Figure 2j). Saline-challenged and nonimmunized OVA-challenged control mice did not show stainable mucus in the bronchiolar epithelium. No thickening of the basal membrane was observed even in mice repeatedly challenged for several days (data not shown).

#### IL-4 Is Detected Only in the BALF

IL-4 and IL-5 were detected in the BALF only at 24 h after challenge. IL-5 levels in the serum increased starting 6 h after challenge (Figure 5).

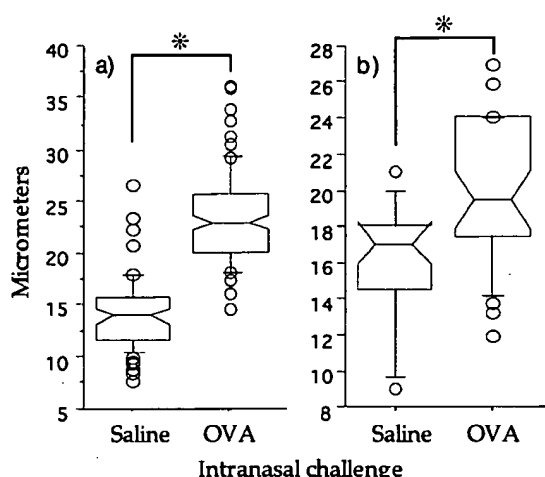
#### BP2 Mice Develop OVA-Induced BHR after a Single Challenge

BP2 mice studied 1 h after the OVA challenge displayed BHR in response to methacholine inhalation, which was over at 3 h, rose at 6 h, and persisted for up to 6 d (Figure 6). Similarly challenged BALB/c mice showed no BHR, except for a small rise at 72 h (data not shown).

#### Inflammatory-Cell Recruitment and Mucous-Cell Metaplasia Are Independent of Each Other in Experimental Allergic Inflammation

We observed no mortality in mice receiving anti-CD3 antibody, but they showed roughened coats and decreased weight. T lymphocytes from mice treated with anti-CD3 were totally, and with the anti-CD4 were partially, depleted in spleens (FACS data, not shown). The anti-CD3 antibody suppressed the OVA-dependent recruitment of eosinophils, lymphocytes, and IgE-bearing PMN (Figure 7), as well as mucus production and BHR in response to methacholine (Figure 8a). When the animals were chal-



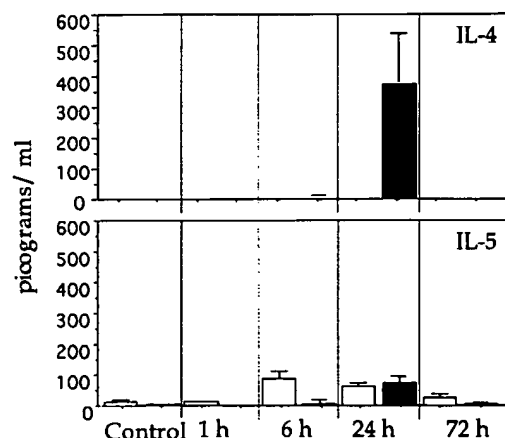


**Figure 4.** Box plot for the height of the epithelium (a) and cross-sectional width (b) of smooth muscle of the major bronchiole of BP2 mice measured 72 h after one intranasal OVA challenge. The left lung lobe was sectioned transversally at around 4 mm from the entry of the bronchi. Measurement was performed at high-power magnification using an image analyzer. \* $P < 0.05$ .

lenged 1 wk after anti-CD3 treatment, infiltration of inflammatory cells was only partly recovered (Figure 7e), and inhibition of BHR persisted (Figure 8b).

The anti-CD4 antibody decreased significantly but did not suppress eosinophil recruitment and mucus production (Figure 9). In this case, the control group treated with multiple injections of rat IgG also showed a reduced number of eosinophils, thus complicating the interpretation. BHR was inhibited by anti-CD4 at 72 h after challenge but not before (Figures 8c and 8d). The infiltration of lymphocytes and the formation of lymphoid follicles were completely inhibited by the anti-CD3 but not by the anti-CD4 antibody (data not shown).

The T-lymphocyte areas of secondary lymphoid organs (spleen and lymph nodes) were depleted of lymphocytes by the anti-CD3 but less so by the anti-CD4 antibody. In the latter case, only focal apoptotic lymphocyte aggregates were



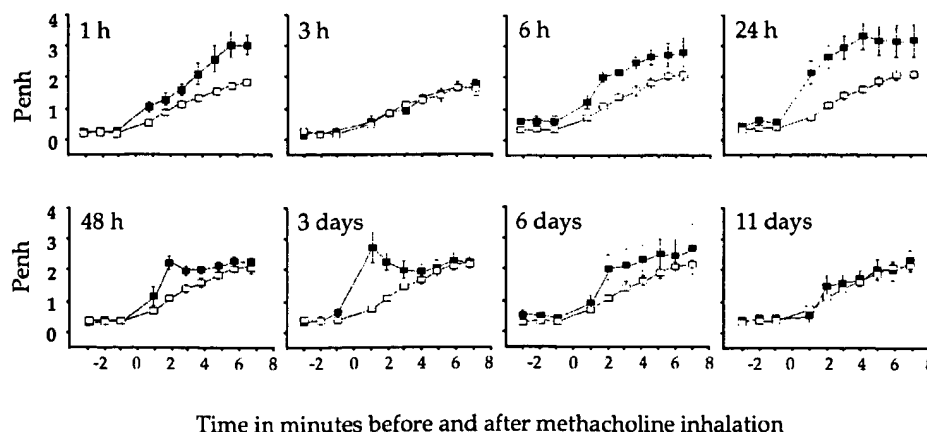
**Figure 5.** IL-4 and IL-5 levels in the serum (open columns) and in the BALF (closed columns) in BP2 mice at different time points after a single intranasal OVA challenge.

seen. Lymphoid organs and the bone marrow of these mice examined 1 wk after anti-CD3 administration showed an intense granulocyte proliferation, particularly of neutrophils, as if the bone marrow was recovering from severe depression.

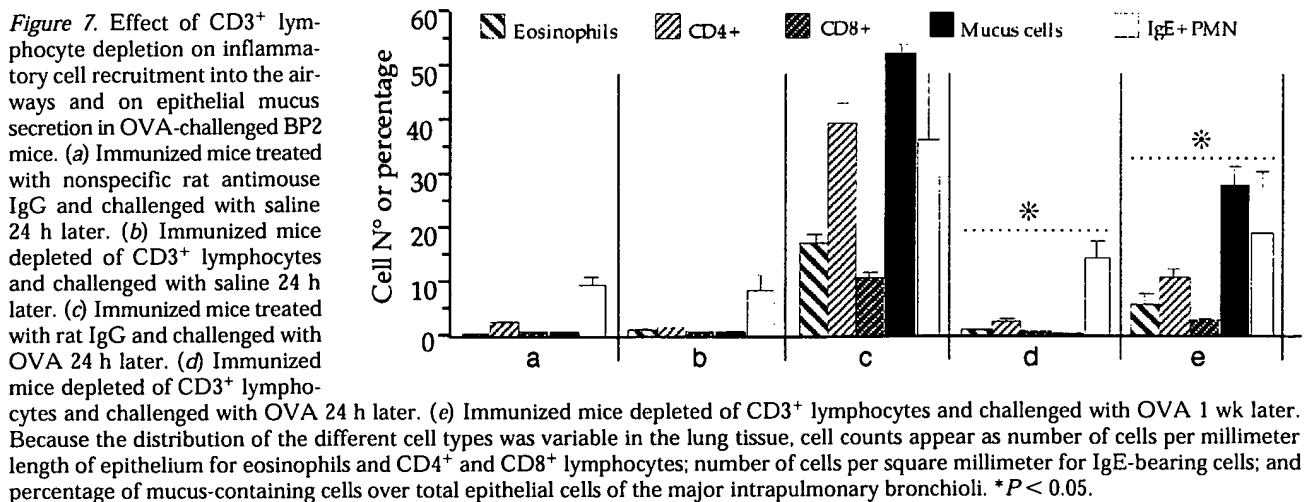
Vinblastine, given at 72 h before challenge, completely suppressed the lung recruitment of eosinophils and of IgE-bearing PMN. It also reduced the number of intravascular neutrophils detected in lung sections to below basal levels (Figure 10). By contrast, vinblastine failed to affect the number of CD4 lymphocytes infiltrating the submucosa. Most importantly, it failed to modify the antigen-induced mucus-cell metaplasia (Figure 3n). Despite complete abrogation of granulocyte recruitment in the lungs, the proportion of mucus-containing cells remained equal to that in the untreated, OVA-challenged BP2 mice.

## Discussion

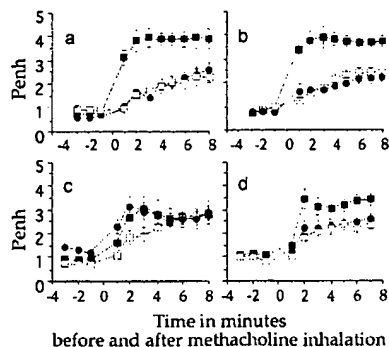
In this study we demonstrate that single antigenic provocations in BP2 mice induce BHR, which lasts up to 6 d, in parallel to eosinophil and lymphocyte recruitment into the airways. As compared with BALB/c mice, BP2 mice



**Figure 6.** Time-dependent BHR in BP2 mice. An average value of the Penh was calculated every minute before and after the methacholine aerosol inhalation. BP2 mice showed early and late antigenic responses similar to those described in human asthma. *Open squares*: immunized mice challenged with saline solution; *closed squares*: immunized mice challenged with OVA.



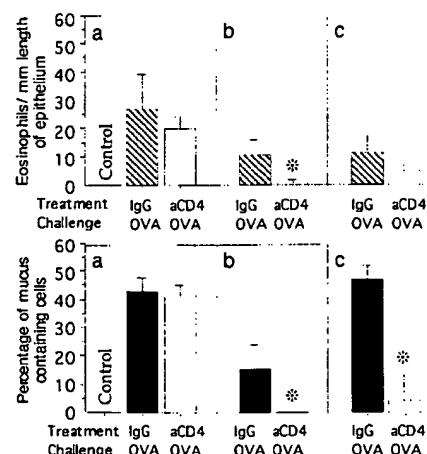
showed a more intense infiltration of eosinophils, lymphocytes, and IgE-bearing PMN in lungs. Eosinophils were located more frequently in the epithelium of BP2 as compared with BALB/c mice, but no associated epithelial sloughing was observed even in multichallenged mice, contrary to what is observed in asthmatic human patients (19). BHR was already noted in BP2 mice 1 h after challenge with OVA, well before the arrival of eosinophils to the lungs. This may relate to the immediate response to antigen, which is attributable to the IgE-dependent release of inflammatory mediators and was confirmed by the identification of partially degranulated mast cells. BHR was over in 3 h, returned at 6 h to last for 6 d, and normalized later (Figure 6). By contrast, BALB/c mice showed a very mild and nonsignificant BHR at 72 h after challenge (not shown). The dual response on BP2 mice recalls the similar kinetics reported for asthmatic human patients (20).



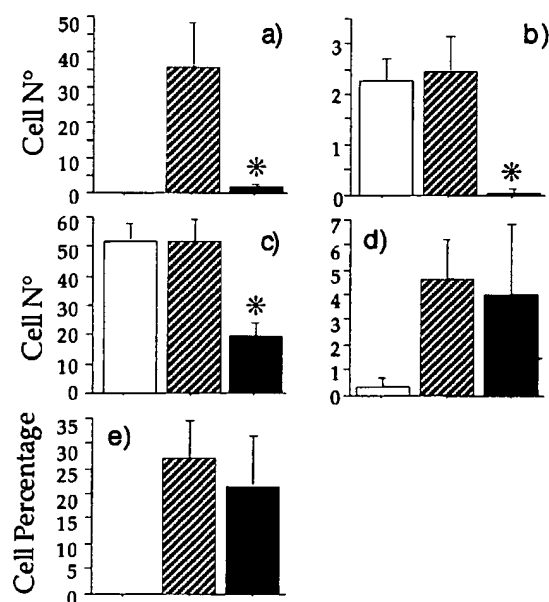
**Figure 8.** Effects of CD3<sup>+</sup> (a and b) and CD4<sup>+</sup> (c and d) lymphocyte depletion on BHR. (a) Results of BHR reading 5 d after anti-CD3 treatment and 72 h after challenges. (b) BHR reading 10 d after anti-CD3 treatment and 24 h after challenges. (c and d) BHR readings 24 and 72 h, respectively, after challenge. *Open squares*: immunized mice challenged with saline solution; *closed squares*: immunized mice challenged with OVA; *closed circles*: immunized and challenged mice that received the depleting antibodies.

Repeated OVA challenges (twice a day for 2 d) in both strains of mice increased the number of eosinophils, lymphocytes, and IgE-bearing PMN infiltrating the lungs, but the BALB/c mice remained nonhyperreactive. In other studies (2) including those of our laboratory (21), multiple antigen sensitizations and challenges in BALB/c mice succeeded in inducing BHR, suggesting that more eosinophils and IgE-bearing PMN are required in the lungs to render BALB/c mice hyperreactive. Cell numbers and the severity of the inflammatory infiltrate, and, as a consequence, the amount of mediators made available, may thus determine whether BHR will be induced.

Antigen-induced eosinophil infiltration in the lungs was completely inhibited in mice depleted of CD3<sup>+</sup> lympho-



**Figure 9.** Effect of anti-CD4 (aCD4) antibody treatment on eosinophil recruitment and epithelial mucus production in BP2 mice. (a) Mice received one injection of 1 mg anti-CD4 antibody and parameters were measured 72 h after OVA challenge. (b) Mice received 300  $\mu$ g of anti-CD4 antibody per day for 3 d and parameters were measured 24 h after OVA challenge. (c) Mice received 300  $\mu$ g of anti-CD4 antibody per day for 3 d and parameters were measured 72 h after OVA challenge. \**P* < 0.05.



**Figure 10.** Effects of vinblastine on OVA-induced recruitment of inflammatory cells and mucous-cell metaplasia in the lungs of mice. *Open column:* immunized mice challenged intranasally with saline solution; *striped columns:* immunized mice challenged intranasally with OVA; *closed columns:* mice that received 5 mg/kg of vinblastine 72 h before the intranasal OVA challenge. Results were read 72 h after OVA challenge. (a) Eosinophils, counted as number of cells per millimeter length of the major bronchiole. (b) IgE-bearing PMN, counted as number of cells per square millimeter of lung section. (c) Neutrophils, counted as number of cells per square millimeter of lung section. (d) CD4<sup>+</sup> lymphocytes, counted as number of cells per millimeter length of the major bronchiole. (e) Mucus-containing epithelial cells in percentage of total epithelial cells of the major bronchiole. \* $P < 0.05$ .

cytes and partially in those receiving the anti-CD4 antibody. Several studies demonstrated that CD4<sup>+</sup> lymphocytes, and particularly those of Th2 subtype, contribute to the infiltration of eosinophils in the lungs by their secretion of eosinophilic cytokines (22). Selective depletion of CD4<sup>+</sup> lymphocytes by the anti-CD4 antibody significantly decreased this infiltration (23). In our study, significant inhibition of eosinophilia and BHR was obtained after three repeated injections of the antibody, but the injection of rat IgG into control mice itself reduced eosinophilia when compared with saline-injected controls. By contrast, administration of a single dose of 200  $\mu$ g anti-CD3 depleted lymphocytes and completely inhibited OVA-induced eosinophilia, IgE-bearing PMN, and BHR. The mechanism of inhibition of eosinophilia by anti-CD3 can be explained by the suppression of T lymphocyte-secreted eosinotactic cytokines. Indeed, MacLean and colleagues (24) demonstrated that anti-CD3 injection into mice inhibited the production of eotaxin and suggested that this was the cause of inhibition of the antigen-induced eosinophilia.

In our studies, we confirmed our previous observation that the eosinophils infiltrating the airways of the OVA-challenged mice are not degranulated (5), contrary to what has been described for human allergic tissues (25). Fur-

thermore, Percoll-collected eosinophils from BALF also were not degranulated. However, while confirming this observation in the tissues examined, we noted granule exocytosis from degenerating eosinophils in the bronchial lumen (Figure 3d). Recently, Persson and Erjefalt (26) suggested that eosinophil lysis and release of free granules may be more important than intact cell degranulation for the release of mediators. Even though we did not observe these alterations in the bronchial submucosa, the presence of degenerating eosinophils and of granule exocytosis in the bronchial lumen supports this hypothesis. The local microenvironment may favor degranulation because the mucus layer of the bronchial lumen is rich in immunoglobulins, especially IgA, that have been shown to degranulate eosinophils *in vitro* (27). Another observation that needs further investigation is the absence of visible lipid bodies either in tissue or in BALF eosinophils. The increase of the numbers and size of lipid bodies in eosinophils is considered a morphologic sign of activation and of the increased synthesis of lipid mediators (28, 29). In comparison with human eosinophils, the absence of lipid bodies in mice in this study implies a possible limitation in the synthesis of lipid mediators of inflammation.

IL-4 and IL-5 are important cytokines for allergy because they promote IgE isotype switch and eosinophilia, respectively (22). Both were detected in the BALF of OVA-challenged mice, and IL-5 was found in the serum as well. The fact that IL-4 was not found in the serum may result from an exclusive local production, for instance by the basophils (30). Staining of cell-bound IgE allowed us to identify mast cells as well as EPO-negative, thus noneosinophilic, non-B lymphocyte IgE-bearing PMN. After confirming their polymorphonuclear morphology in cytospin preparations of BALF from OVA-challenged mice, these cells were identified as basophils by exclusion criteria. As indicated above, these IgE-bearing PMN were recruited to the lungs shortly after the instillation of antigen and, accordingly, well before the massive arrival of eosinophils and lymphocytes. This may have important implications. The arrival of IgE-bearing PMN implies the potential release of cytokines such as IL-4 that play a role in late asthmatic responses. Several observations implicate IgE in the initiation of late responses (31, 32). We (unpublished observations) and others (31, 33) have demonstrated that treating mice with an anti-IgE antibody within the first 24 h before OVA challenge significantly decreases eosinophil recruitment to the lungs. We also demonstrated that this antibody abrogates the antigen-dependent IL-4 secretion into the BALF, highlighting the potential role of IgE in late reactions, through its action on high-affinity IgE receptor-bearing cells such as the IgE-bearing PMN observed in our study. However, recent investigations have shown that IgE-deficient mice are also capable of mounting early- and late-phase reactions to antigen, possibly via the alternative role of IgG<sub>1</sub> in absence of IgE (34).

The absence of modifications in mast-cell numbers after OVA challenge does not imply that they are not activated. Indeed, mast cells were found to have more stainable IgE after OVA challenge in both mouse strains, some of the cells undergoing piecemeal degranulation, as described (35). No increase in mast-cell number has been de-

scribed in tissue from asthmatic human patients (36), but others have noted an increase in tissue (37) and in BALF (38). In our studies, no mast cells, but a significant number of IgE-bearing PMN, were seen in BALF from OVA- and not from saline-challenged control mice. This indicates that IgE-bearing PMN, notably basophils, may play an important role as a source of biologically active substances in mice, in addition to the scarce mast cells present in the mouse airways.

The key difference between BP2 and BALB/c mice after a single OVA challenge lies in the difference in their degrees of pulmonary eosinophilia and of recruitment of IgE-bearing PMN. By contrast, both strains developed mucus-cell metaplasia. Mucus hypersecretion characterizes allergic and nonallergic chronic bronchitis (39–41). In humans, this hypersecretion is due to goblet-cell hyperplasia and probably metaplastic changes of other epithelial cells (42). In this study, we demonstrate that mucus secretion in mice follows metaplastic changes of the nonciliated Clara cells. Normal mice have very few mucus-secreting cells in the tracheobronchial epithelium (43). Following OVA challenge, mucus granules started to form in the apical part of the cytoplasm of Clara cells and gradually increased in number to fill the entire supranuclear cytoplasm by 48 to 72 h. Antigen-dependent metaplastic changes of bronchiolar Clara cells in humans, therefore, may aggravate the mucus secretion and plug small airways, contributing further to bronchoconstriction.

Mucus secretion is favored and induced by several eosinophil and mast-cell products (44). Henderson and associates (45) have shown that leukotriene inhibitors suppress antigen-induced eosinophil infiltration and mucus secretion in the murine lung. In our study, we show that vinblastine treatment abrogates the recruitment of eosinophils and IgE-bearing PMN and significantly reduces that of neutrophils, but does not affect antigen-induced mucus-cell metaplasia of the bronchial epithelium. Therefore, mucous-cell metaplasia of the respiratory epithelium is not directly related to the presence of granulocytes and their products. By contrast, anti-CD3 antibody administered to mice before challenge not only depleted T lymphocytes but also completely inhibited antigen-induced eosinophilia, IgE-bearing PMN recruitment, and mucous-cell metaplasia. Considering these findings together, it can safely be concluded that mucus production is not related to the presence of granulocytes but rather to that of lymphocytes in this model because anti-CD3 antibodies, which inhibited both granulocyte and lymphocyte recruitment into the lungs, also inhibited mucous-cell metaplasia, whereas vinblastine inhibited only granulocyte infiltration in the lungs. A recent study (46) showed that, contrary to previous statements (47), IL-4 is not directly implicated with mucus secretion because transfer of OVA-specific T lymphocytes from IL-4-deficient mice induced mucus production in recipient mice.

Depletion of CD4<sup>+</sup> and CD3<sup>+</sup> lymphocytes inhibited the development of BHR and mucous-cell metaplasia. Vinblastine also inhibited BHR development (unpublished observation), but it did not affect mucous-cell metaplasia. Hyperreactive BP2 mice and normoreactive BALB/c mice showed equivalent mucous-cell metaplasia. Together, these findings suggest that expression of BHR is independent

from this epithelial modification, even though the amount of mucus secreted might contribute to the physical reduction of the bronchial lumen.

Vinblastine was used in our study to inhibit granulocyte recruitment; however, in some cases it has been used to treat the hypereosinophilic syndrome in humans (48, 49). The exact mechanism of inhibition of granulocyte recruitment by vinblastine is not precisely known. Vinblastine induces the formation of paracrystalline aggregates of tubulin, leading to microtubule depolymerization and consequently cell-cycle arrest (50). Inhibition of granulocytes probably results from mitosis arrest leading to the temporary absence of the replenishment of the peripheral granulocyte pool by the bone marrow (51). In addition, the depolymerization of intermediate filaments may compromise cell migration, as has been described for skin Langerhans cells (52). The absence of inhibition of mucus production by vinblastine further supplements the observation that mucus hyperproduction in this model occurs through metaplastic changes of Clara cells rather than through mucous-cell mitosis and hyperplasia.

Additionally, our study shows that the size, topography, and cell composition of the murine airway epithelium and the low number of mast cells resemble the histology of the lower airways of humans and of big mammals. Postmortem and biopsy studies from asthmatic human patients reveal epithelial sloughing, sub-basal fibrosis, and degranulating mast cells and eosinophils, a morphologic marker of activation (53). These lesions were not observed in murine lungs, despite the recruitment of similar inflammatory cells. Murine models of asthma reflect the events taking place in the lower human airways rather than those occurring in the larger bronchi.

In conclusion, we demonstrated that IgE-bearing PMN are recruited to the airways early during the allergic reaction and that their numbers are higher in hyperreactive BP2 mice than in nonhyperreactive BALB/c. This underlines the importance of IgE-stimulated cells in the development of late antigen responses. Mucus hypersecretion is antigen-related and T-lymphocyte-related and was not affected even when eosinophil, IgE-bearing PMN, and neutrophil recruitment was abrogated by vinblastine treatment. Mucus hypersecretion occurred through metaplastic changes of Clara cells in the bronchi and bronchioles, which are not directly related to eosinophils or to their products, as has been suggested previously (54). The implication of mucus for the development of BHR in asthma is frequently stressed, but no direct study on its role is available. Murine models such as ours can contribute to the understanding of the functional mechanisms of BHR, of the mechanisms of epithelial lesions, and of the role of mucus, particularly in the lower airways.

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PATENT

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**Exhibit 3**

## Rac2 is critical for neutrophil primary granule exocytosis

Dalia Abdel-Latif, Melissa Steward, Dawn L. Macdonald, Gordon A. Francis, Mary C. Dinanuer, and Paige Lacy

Neutrophil degranulation is important in many inflammatory disorders, although the intracellular mechanisms underlying this process remain poorly understood. The Rho GTPase, Rac2, has been implicated in control of degranulation in earlier studies. We hypothesized that Rac2 selectively regulates neutrophil primary granule release. Using bone marrow and peritoneal exudate neutrophils from *rac2*<sup>-/-</sup> mice in comparison with similar cells from wild-type C57Bl/6 mice, we found that primary granule myeloperoxidase and elastase release was absent in

*Rac2*<sup>-/-</sup> neutrophils in response to chemoattractant stimulation, cytochalasin B/f-Met-Leu-Phe (CB/fMLP), and CB/leukotriene B<sub>4</sub>. *Rac2*<sup>-/-</sup> neutrophils also failed to exhibit mobilization of the primary granule marker CD63<sup>+</sup> during CB/fMLP stimulation as determined by confocal microscopy. Priming of *Rac2*<sup>-/-</sup> neutrophils with tumor necrosis factor (TNF) or by peritoneal elicitation did not rescue the defect in primary granule release. However, phosphorylation of p38 mitogen-activated protein (MAP) kinase in *Rac2*<sup>-/-</sup> neutrophils was evident in response to

CB/fMLP and/or TNF. Primary granule density and morphology were normal in *Rac2*<sup>-/-</sup> neutrophils. Secondary specific and tertiary granule release, measured by lactoferrin immunoassay and zymography, was normal in response to CB/fMLP and adhesion to fibronectin. These findings suggest an obligatory role for Rac2 in regulation of primary granule release by neutrophils. (Blood. 2004;104:832-839)

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### Introduction

Neutrophils constitute 40% to 80% of circulating white blood cells and are a critical component in the maintenance of innate immunity. Overexuberant activation of neutrophils can lead to extensive degranulation, which may be fatal in septic shock, acute lung injury, and other serious inflammatory disorders. Neutrophils contain 4 different granule populations: primary azurophilic, secondary specific, tertiary granules, and secretory vesicles. These granule types exhibit a hierarchy of release in response to intracellular Ca<sup>2+</sup> spikes,<sup>1</sup> suggesting that granule-specific pathways exist to regulate their secretion. Earlier studies using permeabilized or patch-clamped neutrophils have shown that the final step of granule fusion with membrane, leading to release of granule contents, is dependent on guanosine triphosphate (GTP) and Ca<sup>2+</sup>.<sup>2,3</sup> Determining the identity of GTPase(s) involved in this step has been elusive. Recently, Rho GTPases Rac1, Rac2, and Cdc42 have been suggested as possible exocytotic GTPases.<sup>4</sup>

Rho GTPases, an 18-member subfamily of *ras*-related GTPases, have been shown to be important in regulating a diverse array of cell activation events downstream of receptor activation.<sup>5</sup> Their principle functions are associated with regulation of cytoskeletal reorganization, formation of lamellipodia, chemotaxis, transcriptional events, and cell growth and differentiation.<sup>6</sup> Rac1 and Rac2 exhibit distinct tissue distribution patterns; whereas Rac1 is ubiquitously expressed, Rac2 is limited to hematopoietic cells.<sup>7</sup> Human neutrophils primarily express Rac2 rather than Rac1,<sup>8</sup> whereas murine neutrophils express equivalent levels of Rac1 and

Rac2.<sup>9</sup> Although Rho GTPases are involved in regulating cell functions at different levels, each isoform demonstrates substantial cross-reactivity with each other. Rac1 and Rac2 exhibit considerable homology at the amino acid level (92% identity)<sup>10</sup> and are functionally interchangeable in their ability to activate cytoskeletal remodeling in chemotaxis, as well as superoxide generation through the respiratory burst nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex.<sup>11</sup> Such functional overlap may be partially due to the ability of Rac1 and Rac2 to activate common downstream effectors, such as those containing Cdc42/Rac interactive binding (CRIB) motifs (p21-activating kinases 1-6, among others), and in cascades associated with c-Jun kinase and p38 mitogen-activated protein (MAP) kinase.<sup>12,13</sup> Inhibition of p38 MAP kinase with SB203580 has been demonstrated to partially block f-Met-Leu-Phe (fMLP)-induced primary and secondary granule release in neutrophils, suggesting an important role for p38 MAP kinase in activating receptor-mediated degranulation.<sup>14</sup>

Rac2 and Cdc42 have been implicated in the regulation of degranulation in mast cells.<sup>4,15,16</sup> Addition of active recombinant isoforms of Rac2 and Cdc42 delayed the loss of sensitivity to GTPγS and Ca<sup>2+</sup> during rundown of exocytotic responses in permeabilized rat mast cells,<sup>4</sup> while dominant-negative forms of Cdc42 and Rac1 introduced by vaccinia virus vectors into rat basophilic leukemia (RBL-2H3) cells inhibited antigen-induced secretion.<sup>16</sup> In addition, *Clostridium difficile* toxin B, an inhibitor of Rac1, Rac2, and Cdc42 but not RhoA, blocked FcεRI-mediated degranulation from RBL

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cells.<sup>17,18</sup> However, these studies could not discriminate between pathways selectively regulated by distinct GTPase isoforms. Thus, the use of gene deletion models is essential for defining the role of each specific Rho GTPase isoform in degranulation.

Gene deletion of *Rac2* in mice was shown to lead to a profound loss of chemotactic ability in peripheral blood and bone marrow neutrophils, as well as decreased superoxide production in response to fMLP, tumor necrosis factor (TNF), or phorbol myristate acetate (PMA).<sup>19</sup> Phosphorylation of p38 MAP kinase was slightly decreased in stimulated *Rac2*<sup>-/-</sup> neutrophils,<sup>20</sup> whereas that of ERK1/2 was more significantly diminished.<sup>19</sup>

Recently, a male infant was reported expressing a dominant-negative form of *Rac2* (*Rac2D57N*),<sup>21,22</sup> which led to multiple life-threatening infections. Peripheral blood neutrophils from this child exhibited marked deficiencies in adhesion and respiratory burst, while degranulation assays suggested a defect in primary granule release. However, degranulation responses have not been studied in detail in *Rac2*-deficient neutrophils.

In this study, we hypothesized that *Rac2* is a crucial regulator of neutrophil primary granule release. Peritoneal exudate and bone marrow neutrophils from *rac2*<sup>-/-</sup> mice were harvested to investigate degranulation responses to chemoattractant stimulation. We show an absence of primary granule release, with intact secondary and tertiary granule exocytosis, in *Rac2*<sup>-/-</sup> neutrophils.

## Materials and methods

### Materials

Chemicals were purchased from Sigma-Aldrich (Oakville, ON) unless otherwise noted. Ca<sup>2+</sup>, Mg<sup>2+</sup>, and phenol red-free Hanks balanced salt solution (HBSS) and phosphate-buffered saline (PBS; pH 7.2) were obtained from Life Technologies (Grand Island, NY). HBSS (pH 7.4) was supplemented with bovine serum albumin (BSA; 0.1%) and glucose (1%). Phenol red-free RPMI-1640 was purchased from Life Technologies. Recombinant murine TNF was from BD Pharmingen (San Diego, CA). Antibodies to p38 MAP kinase and phospho-p38 were obtained from BD Biosciences (Mississauga, ON).

### Animals

The *rac2*<sup>-/-</sup> mice used in this study were previously generated by targeted disruption of the *rac2* gene,<sup>19</sup> which had been backcrossed into C57Bl/6 mice for more than 11 generations. Wild-type C57Bl/6 control mice were from Charles River Canada (Saint-Constant, PQ, Canada). Animals were housed under specific pathogen-free conditions and fed autoclaved food and water as needed. Mice used in these experiments were between 8 and 20 weeks of age.

### Preparation of bone marrow and peritoneal neutrophils

Bone marrow neutrophils (BMNs) were obtained by density gradient centrifugation using a modification of previously described techniques.<sup>20,23</sup> Briefly, BMNs were isolated from femurs and tibias flushed with 3 mL HBSS supplemented with BSA and glucose (HBSS-BG) using a 22-gauge needle (Becton Dickinson, San Jose, CA). Cells were pelleted by centrifugation at 300g for 10 minutes at 4°C before mixing with 45% Percoll (Pharmacia, Uppsala, Sweden) and layering onto gradients consisting of 3 mL 81%, 2 mL 62%, 2 mL 55%, and 2 mL 50% Percoll in HBSS-BG. After centrifugation of gradients at 600g for 30 minutes at 10°C, BMNs were removed from the interface between the 62% and 81% layers and washed twice with HBSS-BG. To remove contaminating red blood cells, BMNs were centrifuged on Histopaque 1119 at 600g for 30 minutes at 10°C, and washed twice in HBSS-BG. We obtained 66% to 70% neutrophils for wild-type (WT) and *Rac2*<sup>-/-</sup> bone marrow preparations as determined by Diff-Quik (Fisher Scientific, Nepean, ON, Canada) staining, which is

similar to previously published values.<sup>20</sup> Neutrophil viability was more than 90% as determined by trypan blue exclusion.

Peritoneal exudate neutrophils (PENs) were isolated by intraperitoneal injection of mice with 2 mL 0.05% sodium caseinate in sterile saline 4 hours prior to sacrifice and peritoneal lavage with 6 mL to 8 mL HBSS-BG supplemented with 2 U/mL heparin.<sup>24</sup> Cells were washed with phenol red-free RPMI-1640 and stained with Kimura stain for differential counts. The purity averaged between 88% and 94% for neutrophils from 9 animals, with contaminating mast cells. Injection of animals with sodium caseinate was essential for eliciting PENs, since no PENs could be obtained from uninjected animals.

### Incubation of cells for degranulation assays

PENs and BMNs were washed and resuspended at  $5 \times 10^6$  cells/mL to  $10 \times 10^6$  cells/mL in phenol red-free RPMI-1640 for all degranulation assays, which were carried out on cells in suspension. Aliquots (50  $\mu$ L) of cells were preincubated with 5  $\mu$ g/mL cytochalasin B (CB) for 5 minutes at 37°C before adding increasing doses of fMLP (0  $\mu$ M–40  $\mu$ M) or leukotriene B<sub>4</sub> (LTB<sub>4</sub>; 0  $\mu$ M–1  $\mu$ M). Cells were incubated for an additional 15 minutes or 1 hour before terminating by centrifugation at 4°C for 5 minutes at 300g. Supernatants were removed for assay of released granule proteins, whereas unstimulated cells were lysed by addition of an equivalent volume of RPMI-1640 containing 0.5% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate). For fibronectin adhesion assays, BMNs were incubated on recombinant human fibronectin-coated plates (coated with RetroNectin from PanVera, Fisher Scientific) for 1 hour at 37°C in a humidified incubator as previously described,<sup>25,26</sup> and in some experiments, stimulated for an additional 15 minutes with 5  $\mu$ M fMLP.

### Measurement of myeloperoxidase, elastase, and lactoferrin

Myeloperoxidase (MPO), a marker for primary granules, was assayed by using tetramethylbenzidine (TMB) based on a previously established technique.<sup>27</sup> Briefly, 150  $\mu$ L TMB substrate solution was added to 50  $\mu$ L of sample and incubated (ambient temperature, 30 minutes) prior to termination with 50  $\mu$ L 1 M H<sub>2</sub>SO<sub>4</sub>. Plates were read spectrophotometrically at 450 nm (Molecular Devices, Sunnyvale, CA). Elastase activity was measured by an EnzCheck Elastase assay kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR). Briefly, 100  $\mu$ L fresh sample was incubated with 100  $\mu$ L substrate solution (DQ elastin labeled with BODIPY FL dye; Molecular Probes) for 24 hours at room temperature in the dark (shorter incubations generated less fluorescence) before reading the resulting fluorescence at 505 nm for excitation and 515 nm for emission. Absorbance values for released MPO and elastase were divided into the average of values from 0.5% CHAPS-lysed cells to give percentage of total cellular mediator released.

Lactoferrin (LTF) was assayed by enzyme-linked immunosorbent assay (ELISA) based on a previously published observation demonstrating cross-reactivity of human anti-LTF (Sigma-Aldrich) for murine LTF.<sup>28</sup> Values of release were plotted as optical density, since this assay technique produced low values for total cellular LTF.

### Zymography

Supernatants of resting and stimulated cells were subjected to gel electrophoresis and analyzed for gelatinase activity as previously described.<sup>29</sup> Briefly, 4× zymography loading buffer (40% glycerol, 8% sodium dodecyl sulfate [SDS], 20  $\mu$ g/mL bromophenol blue in 0.25 M Tris, pH 6.8) was added to cell supernatants and the mixture was loaded on 10% acrylamide ready-made zymogram gels (BioRad Laboratories, Hercules, CA). Electrophoresis was carried out at 160 mV for 2 hours and gels were washed 3 times for 20 minutes with 2.5% Triton X-100 at room temperature with shaking. Gels were incubated with zymography buffer (0.9% NaCl, 5  $\mu$ M CaCl<sub>2</sub>, 0.000 25% NaN<sub>3</sub> in 50 mM Tris) for up to 1 week at 37°C. Gel staining was carried out using 0.5% Coomassie blue G-250 in 25% methanol, 10% acetic acid for 1 hour with shaking at room temperature, followed by destaining overnight with 4% methanol and 8% acetic acid.

### Confocal microscopy

Confocal analysis of neutrophil cytopins was carried out as previously reported<sup>30</sup> using 10  $\mu\text{g/mL}$  mouse monoclonal antibody to CD63 (Serotec, Raleigh, NC). Binding of CD63 was detected with 2  $\mu\text{g/mL}$  goat antimouse immunoglobulin G (IgG) secondary antibody conjugated to Alexa 488 (Molecular Probes, Eugene, OR). Confocal images were acquired on a Zeiss LSM510 confocal laser scanning system (Carl Zeiss Imaging, Thornwood, NY) using a 40 $\times$  (1.3 numerical aperture) Plan-Neofluor oil immersion objective.

### Electron microscopic (EM) analysis of BMNs

BMNs were pelleted from WT and *rac2*<sup>-/-</sup> mice and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer before treatment with diaminobenzidine (DAB) solution for 1 hour (0.01% H<sub>2</sub>O<sub>2</sub>, 0.05 M Tris-HCl, pH 6.8, and 5 mg/mL DAB), using a modification of a previously described technique,<sup>31</sup> to enhance staining of peroxidase-containing granules. Samples were submitted to conventional fixation procedure with 1% osmium tetroxide and sequential dehydration steps before addition of Epon resin and sectioning onto uncoated grids. Sections were subjected to a postfix staining step with lead citrate and uranyl acetate, then analyzed (Hitachi model H7000 transmission electron microscope).

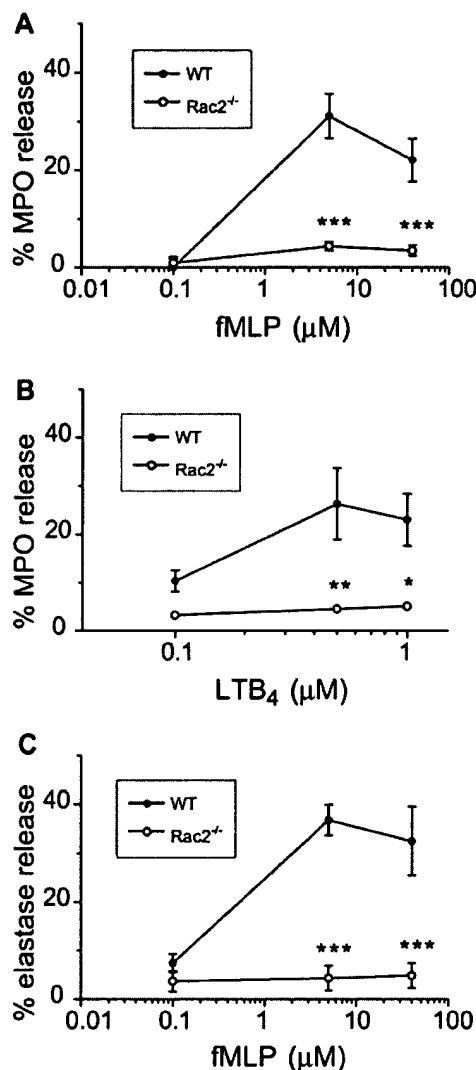
### Data presentation

Release of MPO and elastase was calculated as a percentage of total cellular mediator activity by dividing the corrected absorbance of supernatants into the sum of supernatants and average corrected values for lysed pellets. Data were analyzed by 2-tailed Student *t* test or one-way analysis of variance (ANOVA) analysis followed by Tukey column comparison, and depicted in figures as mean plus or minus the standard error of the mean (SEM). All figures shown represent averages of at least 3 separate experiments.

## Results

### Rac2 is essential for primary granule release in response to chemoattractants

Stimulation of neutrophils *in vitro* with cytochalasin B in combination with chemoattractants results in release of primary granule contents, which include mediators that are crucial for microbial killing.<sup>32</sup> We tested the effect of CB/fMLP on primary granule release from freshly prepared WT and *Rac2*<sup>-/-</sup> BMNs by measuring the release of MPO. As shown in Figure 1A, MPO release from WT neutrophils was detected in response to 5  $\mu\text{M}$  to 40  $\mu\text{M}$  fMLP stimulation, with maximal release at 5  $\mu\text{M}$  fMLP (31%  $\pm$  2%). In contrast, *Rac2*<sup>-/-</sup> BMNs were defective in their ability to release primary granule MPO during CB/fMLP stimulation. Maximal MPO release from *Rac2*<sup>-/-</sup> neutrophils was 4.4%  $\pm$  0.5% (5  $\mu\text{M}$  fMLP), which did not significantly differ from baseline values obtained in unstimulated *Rac2*<sup>-/-</sup> BMNs. WT and *Rac2*<sup>-/-</sup> BMNs treated with CB alone did not release significant levels of MPO (2.6%  $\pm$  1.9% and 0.8%  $\pm$  0.8%, respectively). The MPO content in lysed unstimulated cells was not significantly different between WT and *Rac2*<sup>-/-</sup> BMNs (cell pellets averaged 0.34  $\pm$  0.04 versus 0.46  $\pm$  0.23 optical density [OD], respectively), suggesting that MPO was synthesized and stored at similar levels in *Rac2*<sup>-/-</sup> BMNs as those of WT BMNs. Similar release profiles were obtained with BMNs stimulated by dihydrocytochalasin B (5  $\mu\text{g/mL}$ ) and 0.1  $\mu\text{M}$  to 40  $\mu\text{M}$  fMLP (data not shown), suggesting that preincubation with CB did not adversely affect secretion because of interference with glucose transport. Similarly, increasing doses of another chemoattractant, LTB<sub>4</sub>, induced the release of MPO from WT BMNs but not *Rac2*<sup>-/-</sup> BMNs (Figure 1B).

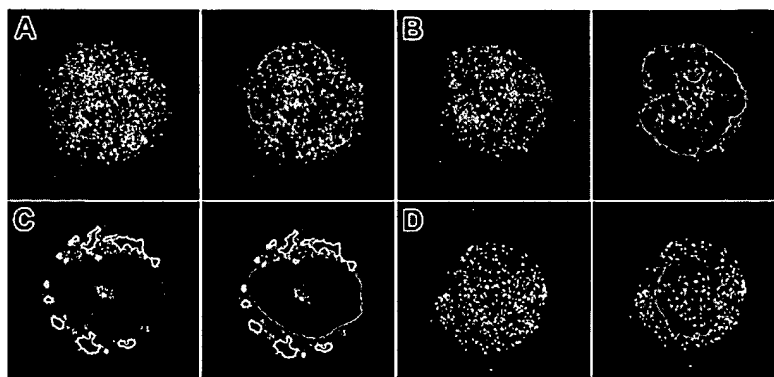


**Figure 1. Absence of primary granule release in chemoattractant-stimulated *Rac2*<sup>-/-</sup> neutrophils.** Freshly prepared BMNs from WT and *rac2*<sup>-/-</sup> neutrophils were examined for their ability to release the primary granule marker MPO in response to increasing doses of fMLP (A) for 15 minutes following 5 minutes preincubation with 5  $\mu\text{g/mL}$  CB. MPO release was determined and shown as percentage of total cellular lysate values. (B) Neutrophils were stimulated with increasing doses of LTB<sub>4</sub> for 1 hour following 5 minutes preincubation with 5  $\mu\text{g/mL}$  CB. (C) Elastase activity in supernatants of neutrophils stimulated with CB/fMLP. \**P* < .05. \*\**P* < .02. \*\*\**P* < .01. Error bars indicate SEM.

Addition of 50 U/mL superoxide dismutase did not enhance CB/fMLP-induced secretion of MPO, suggesting that released MPO was not significantly inactivated by MPO-catalyzed oxidation.<sup>33</sup> These findings suggest that *Rac2*<sup>-/-</sup> neutrophils exhibit a deficiency in MPO release in response to stimulation by 2 different agonists.

We were unable to obtain significant MPO release in WT or *Rac2*<sup>-/-</sup> BMNs in response to murine recombinant TNF (50 ng/mL, 5 minutes) and fMLP (0  $\mu\text{M}$ –40  $\mu\text{M}$ , 15 minutes; data not shown). Fibronectin adhesion (1 hour) induced insignificant MPO release from WT (5%  $\pm$  2%) and *Rac2*<sup>-/-</sup> (0.2%  $\pm$  0.15%) BMNs. Addition of fMLP (0  $\mu\text{M}$ –40  $\mu\text{M}$ ) to fibronectin-adhered BMNs did not enhance MPO release in WT (4.8%  $\pm$  0.5%) or *Rac2*<sup>-/-</sup> (0.8%  $\pm$  0.75%) BMNs. This is in contrast to human neutrophils, which release elastase in response to fibronectin.<sup>34</sup> These findings suggest that cytochalasin B pretreatment is necessary to induce

**Figure 2. CD63<sup>+</sup> granule translocation is inhibited in *Rac2*<sup>-/-</sup> neutrophils.** Cytospins were prepared from freshly isolated BMNs treated with CB/fMLP (5  $\mu$ M) for 15 minutes, stained with 10  $\mu$ g/mL anti-CD63 as a primary granule marker (green), and counterstained with DAPI (blue) to show nuclear structure. Panels show representative images from (A) resting WT BMNs, (B) resting *Rac2*<sup>-/-</sup> BMNs, (C) CB/fMLP-stimulated WT BMNs, and (D) CB/fMLP-stimulated *Rac2*<sup>-/-</sup> BMNs, with the left panel in each case showing CD63 alone and the right panel showing combined images of CD63 immunoreactivity and DAPI nuclear stain. Original magnification,  $\times 40$ .



primary granule release from neutrophils, as supported by previous studies.<sup>32,35</sup> We also tested the effect of 2  $\text{Ca}^{2+}$  ionophores, A23187 and ionomycin, on MPO release from BMNs, but these failed to induce significant release from WT or *Rac2*<sup>-/-</sup> cells at 1  $\mu$ M to 10  $\mu$ M (data not shown). The inability of murine BMNs to release MPO in response to these stimuli may indicate a species difference, since human neutrophils release primary granule products in response to similar conditions (data not shown).<sup>32,34,36</sup>

In order to confirm that primary granule mediator release was deficient in *Rac2*<sup>-/-</sup> BMNs, we measured elastase activity as an additional primary granule marker. Elastase release was also abolished in CB/fMLP-stimulated *Rac2*<sup>-/-</sup> BMNs compared with WT cells (Figure 1C). Similar results were obtained in cells stimulated with CB/LTB<sub>4</sub> (data not shown). These observations suggest that *Rac2* gene deletion leads to loss of primary granule mediator release in neutrophils.

The responsiveness of *Rac2*<sup>-/-</sup> neutrophils to respiratory burst stimulation was confirmed by testing BMNs from *rac2*<sup>-/-</sup> and WT mice using a conventional cytochrome *c* reduction assay. BMNs from *Rac2*<sup>-/-</sup> mice generated 53% less  $\text{O}_2^-$  than WT controls in response to stimulation with 500 ng/mL PMA ( $0.51 \pm 0.07$  compared with  $1.1 \pm 0.2$  nmol  $\text{O}_2^-/10^6$  cells/min, respectively;  $P < .05$ ). These values correlate with those of previously published data<sup>19</sup> and suggest that these animals are genetically and phenotypically similar to those previously studied.

#### CD63 translocation in *Rac2*<sup>-/-</sup> neutrophils is deficient in response to CB/fMLP

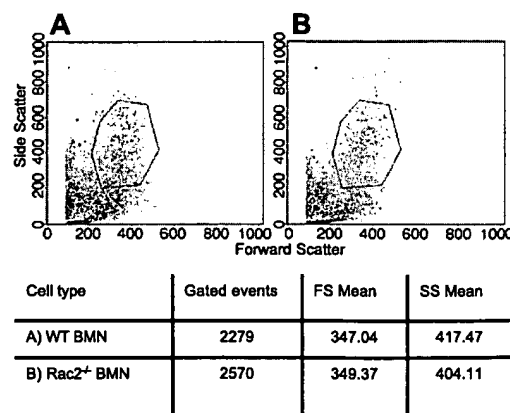
To determine if primary granules translocate to the membrane in response to CB/fMLP in *Rac2*<sup>-/-</sup> neutrophils, cytopins of resting and stimulated WT and *Rac2*<sup>-/-</sup> BMNs were prepared and labeled for CD63 immunoreactivity. Resting WT and *Rac2*<sup>-/-</sup> BMNs exhibited a homogeneous cytoplasmic staining, suggesting the presence of primary granules in the cytoplasm (Figure 2A-B). Following stimulation with CB/fMLP (5  $\mu$ M, 15 minutes) of WT BMNs, large aggregates of CD63 immunoreactivity formed in submembranous regions, suggesting that CD63<sup>+</sup> granules had translocated to the cell membrane (Figure 2C). In contrast, *Rac2*<sup>-/-</sup> BMNs did not display any change in CD63 labeling from that of resting cells (Figure 2D), suggesting that granule trafficking may be deficient in *Rac2*<sup>-/-</sup> neutrophils. It was not possible to resolve the structure of individual CD63<sup>+</sup> granules by confocal analysis, as these are smaller than the limit of resolution of this technique ( $\sim 0.3$   $\mu$ m in diameter).<sup>3</sup>

#### Granular morphology in WT and *Rac2*<sup>-/-</sup> neutrophils is identical

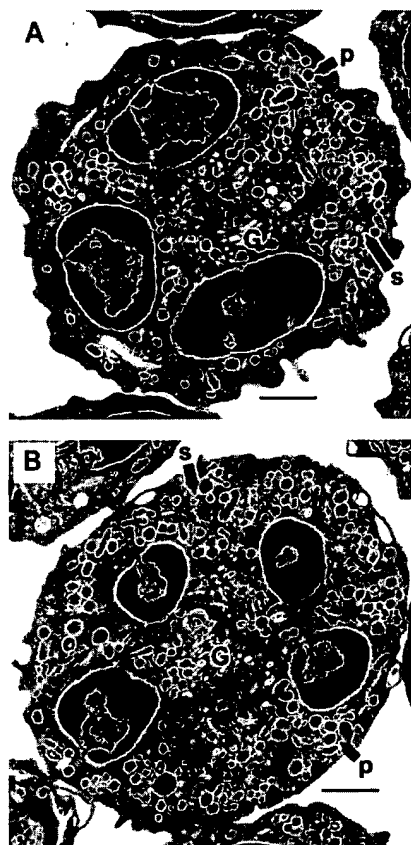
Changes in granule morphology in *Rac2*<sup>-/-</sup> neutrophils may explain the defect in primary granule release, such as an overall reduction in granule volume or numbers. To test whether neutrophil morphology from *rac2*<sup>-/-</sup> mice was altered in comparison with WT cells, BMNs were subjected to analysis by flow cytometry and assessed for their size and granularity. There were no significant differences in mean forward and side scatter values obtained from gated BMNs from WT and *rac2*<sup>-/-</sup> mice (Figure 3), suggesting that cell sizes and granularity of BMNs were similar. BMNs from WT and *rac2*<sup>-/-</sup> animals were further analyzed by EM using a conventional osmium tetroxide-based fixation technique coupled with DAB staining to enhance the electron density of peroxidase-containing organelles. Both WT and *Rac2*<sup>-/-</sup> neutrophils exhibited identical granule morphology in BMNs (Figure 4), with no apparent difference in granule volume, numbers, or electron density. The similarity in cellular morphology was evident in all cells observed. These findings suggest that *rac2* gene deletion did not affect granulogenesis in BMNs, or any other apparent aspects of cellular morphology in resting cells.

#### Loss of fMLP-induced primary granule release in *Rac2*<sup>-/-</sup> neutrophils is not rescued by in vitro priming with TNF

In order to determine if in vitro priming may reconstitute the release of primary granule release in *Rac2*<sup>-/-</sup> neutrophils, TNF was



**Figure 3. Similar granularity in WT and *Rac2*<sup>-/-</sup> neutrophils.** BMNs were isolated from (A) WT and (B) *Rac2*<sup>-/-</sup> mice and subjected to flow cytometric analysis to analyze their side scatter properties. Each scatter plot shows 10 000 events. Gates in the scatter plots are arbitrarily selected to show representative means of forward scatter (FS) and side scatter (SS), as indicated in the table beneath the panels.



**Figure 4. Normal granular morphology in neutrophils from *Rac2*<sup>-/-</sup> mice.** Neutrophils were pelleted from bone marrow and sectioned for EM analysis. Panels show typical cells from (A) WT and (B) *Rac2*<sup>-/-</sup> mice. Labels indicate primary granule (p), secondary granule (s), and Golgi apparatus (G). The bar indicates 1  $\mu$ m. Original magnification,  $\times 7000$ .

preincubated with cells prior to their activation with CB/fMLP. Pretreatment of *Rac2*<sup>-/-</sup> BMNs with TNF (50 ng/mL) failed to correct the defect in MPO release following CB/fMLP stimulation (Figure 5A). Separate experiments were done to confirm that *Rac2*<sup>-/-</sup> neutrophils were responsive to TNF stimulation by carrying out flow cytometric analysis for surface expression of adhesion markers CD11b and CD18. In *Rac2*<sup>-/-</sup> neutrophils stimulated by TNF at 50 ng/mL for 10 minutes, CD11b and CD18 were up-regulated to a similar degree as that seen in WT neutrophils, indicating that cells were effectively primed by TNF incubation (data not shown).

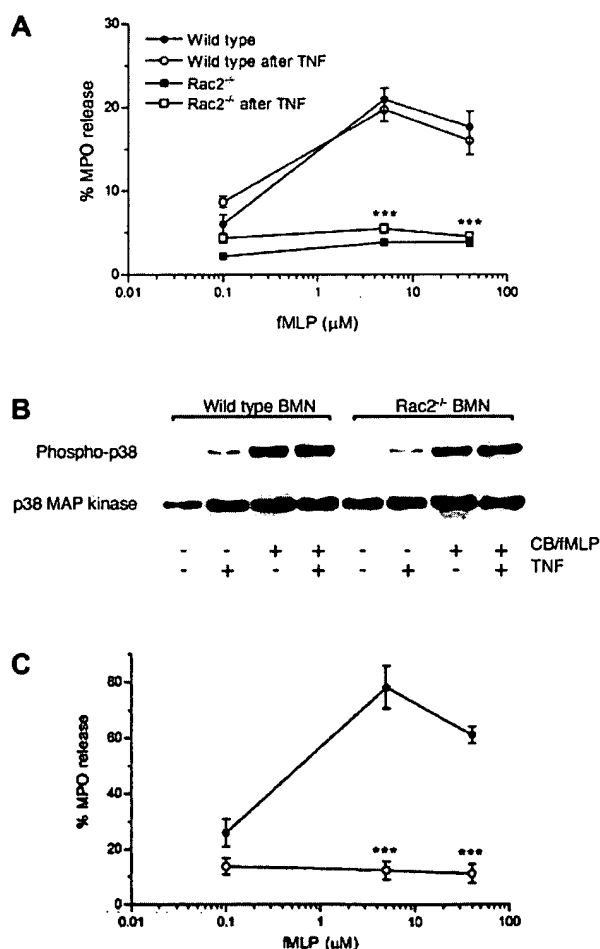
#### p38 MAP kinase phosphorylation in *Rac2*<sup>-/-</sup> neutrophils is induced by TNF and CB/fMLP

TNF is a potent stimulus for p38 MAP kinase phosphorylation in human neutrophils, and may be required for fMLP-induced primary and secondary granule release as well as interleukin 8 (IL-8) secretion.<sup>14,37</sup> We determined whether TNF might also activate p38 kinase in murine BMNs in order to prime these cells for degranulation. Phosphorylation of p38 was investigated in WT and *Rac2*<sup>-/-</sup> BMNs before and after stimulation with TNF (50 ng/mL, 5 minutes), CB/fMLP, and TNF plus CB/fMLP (Figure 5B). In WT neutrophils, TNF and CB/fMLP induced p38 phosphorylation, although TNF was less potent in this regard. This is in contrast to human neutrophils, where TNF was shown to be a potent inducer of p38 phosphorylation.<sup>37</sup> Addition of TNF to CB/fMLP did not

enhance phosphorylation in response to CB/fMLP. Interestingly, *Rac2*<sup>-/-</sup> neutrophils exhibited nearly normal levels of p38 phosphorylation in response to TNF and/or CB/fMLP compared with WT cells. These findings suggest that p38 MAP kinase activation alone is not sufficient for triggering primary granule exocytosis.

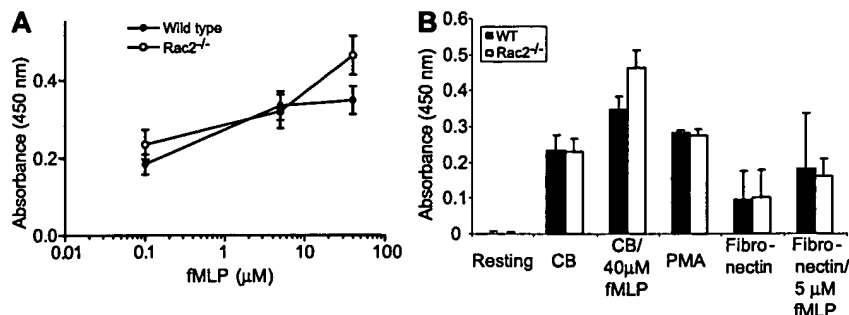
#### Defect in fMLP-induced primary granule release is not due to an in vivo priming deficiency in *Rac2*<sup>-/-</sup> neutrophils

In vivo priming of neutrophils through peritoneal elicitation is likely to be a stronger stimulus for cell priming than in vitro treatment with cytokines. In peritoneal lavage cells, WT neutrophils exhibited a significant dose-dependent release of primary granule MPO in response to CB/fMLP, with responses reaching maximal values at 5  $\mu$ M fMLP (78%  $\pm$  4% of total cellular MPO), as shown in Figure 5C. In contrast, PENs from *Rac2*<sup>-/-</sup> mice demonstrated a lack of MPO release under similar conditions (Figure 5C). *Rac2*<sup>-/-</sup> PENs showed no increase above baseline levels of spontaneous MPO release (14%  $\pm$  2%) in response to all



**Figure 5. Priming of *Rac2*<sup>-/-</sup> neutrophils fails to reverse the degranulation defect.** WT and *Rac2*<sup>-/-</sup> BMNs were stimulated with CB/fMLP (5  $\mu$ M) with or without preincubation of 50 ng/mL TNF for 5 minutes. (A) MPO release from WT and *Rac2*<sup>-/-</sup> BMNs in the presence or absence of TNF preincubation. \*\*\*  $P < .01$  compared with TNF-preincubated WT controls at the same doses of fMLP. (B) Phosphorylation of p38 MAP kinase in *Rac2*<sup>-/-</sup> neutrophils is normal in response to TNF and CB/fMLP. Western blot analysis was carried out for phospho-p38 and p38 MAP kinase in cellular lysates. (C) PENs were treated with increasing doses of fMLP for 15 minutes following 5 minutes preincubation with 5  $\mu$ g/mL CB. MPO release was determined and shown as percentage of total cellular lysate values. \*\*\*  $P < .01$ . Error bars indicate SEM.

**Figure 6. Secondary granule LTF release is equivalent in WT and *Rac2*<sup>-/-</sup> neutrophils.** BMNs were stimulated with increasing doses of (A) fMLP (after CB preincubation). Release was significant ( $P < .05$ ) at the highest dose of agonist. (B) Comparison of the effects of CB, CB with 40  $\mu$ M fMLP, PMA (500 ng/mL), fibronectin adhesion (1 hour), or fibronectin (1 hour) followed by 5  $\mu$ M fMLP on LTF release determined by immunoassay. Error bars indicate SEM.



concentrations of fMLP tested in this study. MPO release from WT PENs was considerably greater than that of WT BMNs, presumably due to their primed state. These observations rule out the possibility that the loss of primary granule release may be due to a priming defect in *Rac2*<sup>-/-</sup> neutrophils.

#### Secondary granule release is normal in *Rac2*<sup>-/-</sup> neutrophils

We next tested whether the loss of Rac2 function might also affect the release of another important granule population in neutrophils, secondary granules, which release bacteriostatic mediators such as LTF during phagocytosis and degranulation. The release of LTF from BMNs was measured in response to stimulation with CB/fMLP. There was normal release of LTF from *Rac2*<sup>-/-</sup> BMNs in response to CB/fMLP stimulation (Figure 6A), which was equivalent to that of WT neutrophils. However, treatment of WT and *Rac2*<sup>-/-</sup> BMNs with CB alone resulted in significant release of LTF ( $0.23 \pm 0.04$  OD for WT and *Rac2*<sup>-/-</sup> cells, after subtracting background of untreated resting cells). This represented between 50% and 67% of the total releasable LTF in response to maximal stimulation (40  $\mu$ M fMLP). To determine the release of LTF under more physiologic conditions, freshly prepared BMNs were adhered to fibronectin-coated plates for 1 hour and their supernatants assayed for LTF release. Both WT and *Rac2*<sup>-/-</sup> BMNs released LTF following adhesion, representing approximately 24% to 26% of the total releasable LTF (Figure 6B). This release was not significantly enhanced by addition of increasing doses of fMLP (0  $\mu$ M-40  $\mu$ M) or coincubation with TNF (10 ng/mL). Addition of 500 ng/mL PMA induced approximately 59% to 81% of total releasable LTF, which did not differ in WT or *Rac2*<sup>-/-</sup> BMNs, suggesting that LTF secretion in response to protein kinase C (PKC) activation is similar in both WT and *Rac2*<sup>-/-</sup> BMNs. In our hands, this assay was relatively insensitive for detecting LTF release. However, we were able to determine that there was no significant difference in LTF release in WT and *Rac2*<sup>-/-</sup> BMNs, indicating that this pathway of exocytosis is independent of Rac2.

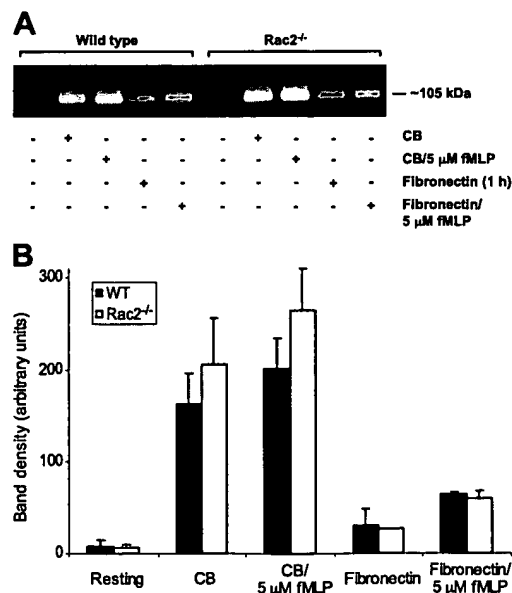
#### Tertiary granule release is not affected in *Rac2*<sup>-/-</sup> neutrophils

Supernatants of CB/fMLP-stimulated WT and *Rac2*<sup>-/-</sup> BMNs were subjected to zymography analysis to determine the release of gelatinase. Unstimulated cells released negligible quantities of gelatinase activity, whereas cells stimulated with CB alone or CB plus 5  $\mu$ M fMLP released a significant amount of gelatinase activity migrating at approximately 105 kDa (Figure 7A). This molecular weight corresponds with the expected size of murine matrix metalloproteinase-9 (MMP-9),<sup>38</sup> a marker for the tertiary granules in neutrophils.<sup>1</sup> Stimulation of BMNs with CB/fMLP did not enhance the release of MMP-9 over CB alone (Figure 7). Total cell MMP-9 was equivalent in WT and *Rac2*<sup>-/-</sup> BMNs based on

densitometric analysis of zymography bands from cell lysates, suggesting that *Rac2*<sup>-/-</sup> BMNs were not deficient in MMP-9 synthesis and storage (data not shown). Similarly to LTF release, CB alone induced significant gelatinase release in BMNs from WT and *rac2*<sup>-/-</sup> mice (78%-80% of total releasable MMP-9). However, we found that MMP-9 release occurred under physiologic conditions in WT and *Rac2*<sup>-/-</sup> BMNs in response to adhesion to fibronectin alone (1 hour) and fibronectin (1 hour) plus 5  $\mu$ M fMLP (Figure 7). Addition of fMLP enhanced the release of MMP-9 when added to fibronectin-adhering cells. Cells released between 22% and 32% of maximal MMP-9 release in response to fibronectin adhesion followed by fMLP (5  $\mu$ M) stimulation for 15 minutes.

## Discussion

Rho GTPases are critical regulators of cellular activation events, which modulate the function of important intracellular effector molecules.<sup>5</sup> In this report, we show that Rac2 is required for primary granule release in neutrophils in response to chemoattractant stimulation. *Rac2*<sup>-/-</sup> neutrophils exhibited a profound defect



**Figure 7. Release of tertiary granule MMP-9 is similar in WT and *Rac2*<sup>-/-</sup> neutrophils.** (A) Supernatants of WT and *Rac2*<sup>-/-</sup> BMNs, either resting or stimulated with CB, CB with 5  $\mu$ M fMLP, fibronectin (1 hour), or fibronectin (1 hour) with 5  $\mu$ M fMLP, were separated by gel electrophoresis and analyzed by zymography to detect gelatinase activity, most of which migrated at approximately 105 kDa. (B) Graph showing the average optical density of MMP-9 activity in supernatants of cells stimulated by CB, CB with 5  $\mu$ M fMLP, fibronectin adhesion (1 hour), or fibronectin (1 hour) followed by 5  $\mu$ M fMLP. Error bars indicate SEM.

in their ability to release 2 different primary granule mediators, MPO and elastase, in response to CB/fMLP and CB/LTB<sub>4</sub>. In support of these findings, CD63<sup>+</sup> granules in Rac2<sup>-/-</sup> neutrophils failed to translocate in response to CB/fMLP, whereas WT cells exhibited intense granule mobilization to submembranous regions. Addition of the potent priming cytokine TNF did not rescue the degranulation defect in Rac2<sup>-/-</sup> neutrophils in response to CB/fMLP, although studies have shown that TNF augmented PMA-induced superoxide release and fMLP-induced F-actin formation in these cells.<sup>19</sup> Peritoneal exudate neutrophils from *rac2*<sup>-/-</sup> animals also showed no MPO release upon stimulation with CB/fMLP. Therefore, the defect in degranulation is not related to deficiencies in neutrophil priming. This is also the first report, to our knowledge, that describes primary granule mediator release in murine neutrophils.

The loss of primary granule release in Rac2<sup>-/-</sup> BMNs is unrelated to granulogenesis defects, since their side scatter properties resembled that of WT BMNs, and thin sections of Rac2<sup>-/-</sup> BMNs examined by EM exhibited identical granularity to that of WT cells. Therefore, Rac2 appears to be specifically required for the intracellular machinery dedicated to neutrophil primary granule translocation prior to docking and fusion. Granule translocation is likely to be dependent on highly specific cytoskeletal remodeling events. Moreover, the release of secondary and tertiary granules was not defective in CB/fMLP-treated Rac2<sup>-/-</sup> neutrophils, suggesting that cytoskeletal reorganization associated with exocytosis in these granule populations was intact in these cells. The addition of CB (an inhibitor of actin filament formation that acts as a priming reagent in neutrophils) is a prerequisite for chemoattractant-induced primary granule release. However, CB alone was able to induce secondary and tertiary granule release, indicating that degranulation can occur by CB-induced microfilament depolymerization.<sup>39</sup> Since CB is a nonphysiologic agent, we carried out measurements of LTF and MMP-9 release in response to adhesion to fibronectin and fMLP, and found that the release of secondary and tertiary granules was similar in WT and Rac2<sup>-/-</sup> BMNs. Our findings suggest that secondary and tertiary granule exocytosis is unimpeded in Rac2<sup>-/-</sup> BMNs during physiologic stimulation. Although it was not possible to compare the release of mediators from all 3 granule types in response to the same stimulus, the data show that primary granule release in Rac2<sup>-/-</sup> neutrophils was abolished even during artificial or physiologic priming.

These findings also indicate a lack of functional overlap between Rac1 and Rac2 in neutrophil primary granule exocytosis. Another study has recently suggested divergent roles for Rac1 and Rac2 in cell spreading and motility, respectively.<sup>40</sup> This is in contrast to previous studies which suggested overlapping roles for Rho GTPases in cytoskeletal reorganization, cell motility, and gene transcription.<sup>5</sup> Murine BMNs from *rac2*<sup>-/-</sup> animals express Rho, Rac1, and Cdc42,<sup>19</sup> as determined by Western blot analysis (data not shown), and generate similar amounts of Rac1 and Rac2,<sup>9</sup> in contrast to human neutrophils which express predominantly Rac2.<sup>8</sup> No other Rho GTPase substituted for Rac2 in mediating primary granule release in spite of being expressed at levels similar to WT neutrophils. Thus, downstream signals of Rac2 in this pathway are unlikely to cross-react with other GTPases. Based on our experimen-

tal data reported in this paper, we were unable to discriminate whether the defect in primary granule release in Rac2<sup>-/-</sup> neutrophils was in the signaling pathway adjacent to the receptor or in the final steps leading up to exocytosis, which will be important to determine in future investigations.

Rac2 has been shown to activate MAP kinases in other cell types.<sup>41</sup> In particular, p38 and ERK1/2 kinases have been proposed to act as effectors downstream of Rac2 signaling during fMLP stimulation. Moreover, studies have demonstrated that p38 MAP kinase is an obligatory effector molecule required for fMLP-induced IL-8 release in human neutrophils,<sup>37</sup> and that inhibition of p38 MAP kinase by SB203980 led to decreased primary and secondary granule release in CB/fMLP-stimulated human neutrophils.<sup>14</sup> Similarly, p38 MAP kinase activity was slightly decreased in fMLP-stimulated Rac2<sup>-/-</sup> neutrophils, suggesting that p38 MAP kinase may function downstream of Rac2.<sup>19</sup> In this study, we found that CB/fMLP induced strong p38 phosphorylation in Rac2<sup>-/-</sup> BMNs, whereas TNF did not augment the phosphorylation signal induced by CB/fMLP. These findings suggest that activation of p38 MAP kinase is not related to the degranulation defect in Rac2<sup>-/-</sup> neutrophils, and that p38 activation may not be sufficient for primary granule release.

In contrast to the effects of *rac2*<sup>-/-</sup> gene deletion on primary granule release, secondary and tertiary granule release was intact in Rac2<sup>-/-</sup> BMNs in response to CB/fMLP and adhesion to fibronectin. In addition, PMA induced LTF release from both WT and Rac2<sup>-/-</sup> BMNs. The diacylglycerol-sensitive C1 domain serves as a target for phorbol ester stimulation, and proteins containing this domain include conventional isoforms of PKC.<sup>42</sup> PMA induces superoxide production from neutrophils by activation of the NADPH oxidase complex through PKC stimulation.<sup>43,44</sup> However, PMA did not evoke primary granule release from murine bone marrow neutrophils. The discrepancy in the effects of PMA on superoxide production and primary granule exocytosis, and the observation that Rac2 is important in both processes, suggests that diacylglycerol-sensitive proteins, such as conventional PKC isoforms, may represent a split in signaling pathways downstream of Rac2 that have not yet been identified for primary granule exocytosis.

In summary, these findings indicate that Rac2 is a critical regulatory GTPase in primary granule exocytosis. The morphology of neutrophils from *rac2*<sup>-/-</sup> mice was similar to that of WT animals, suggesting that granulogenesis was normal in these cells. Priming of neutrophils and expression of other homologous Rho GTPases in these cells failed to substitute for the degranulation defect. These findings provide important insights into pathways regulating neutrophil exocytosis, which may serve as targets for anti-inflammatory therapy.

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Examiner: Maher M. Haddad

Filed: March 17, 2004

Group Art Unit: 1644

For: *Blocking Peptide for Inflammatory Cell Secretion*

Date: October 31, 2005

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## Exhibit 4



# Divergence of Mechanisms Regulating Respiratory Burst in Blood and Sputum Eosinophils and Neutrophils from Atopic Subjects<sup>1</sup>

Paige Lacy,<sup>2</sup> Dalia Abdel Latif, Melissa Steward, Sorin Musat-Marcu, S. F. Paul Man, and Redwan Moqbel

Eosinophil respiratory burst is an important event in asthma and related inflammatory disorders. However, little is known concerning activation of the respiratory burst NADPH oxidase in human eosinophils. Conversely, neutrophils are known to assemble NADPH oxidase in intracellular and plasma membranes. We hypothesized that eosinophils and neutrophils translocate NADPH oxidase to distinct intracellular locations, consistent with their respective functions in O<sub>2</sub><sup>-</sup>-mediated cytotoxicity. PMA-induced O<sub>2</sub><sup>-</sup> release assayed by cytochrome *c* was 3.4-fold higher in atopic human eosinophils than in neutrophils, although membrane-permeable dihydrorhodamine-123 showed similar amounts of release. Eosinophil O<sub>2</sub><sup>-</sup> release was dependent on Rac, in that it was 54% inhibited by *Clostridium difficile* toxin B (400–800 ng/ml). In eosinophils stimulated with PMA, a pronounced shift of cytosolic Rac to p22<sup>phox</sup>-positive plasma membrane was observed by confocal microscopy, whereas neutrophils directed Rac2 mainly to intracellular sites coexpressing p22<sup>phox</sup>. Similarly, ex vivo sputum eosinophils from asthmatic subjects exhibited predominantly plasma membrane-associated immunoreactivity for Rac, whereas sputum neutrophils exhibited cytoplasmic Rac2 staining. Thus, activated sputum eosinophils, rather than neutrophils, may contribute significantly to the pathogenesis of asthma by extracellular release of tissue-damaging O<sub>2</sub><sup>-</sup>. Our findings suggest that the differential modes of NADPH oxidase assembly in these cells may have important implications for oxidant-mediated tissue injury. *The Journal of Immunology*, 2003, 170: 2670–2679.

**R**espiratory burst is an important event in many inflammatory conditions, characterized by production of superoxide anion (O<sub>2</sub><sup>-</sup>) and related reactive oxygen species (ROS),<sup>3</sup> including H<sub>2</sub>O<sub>2</sub> and OH<sup>-</sup> (1). Eosinophils from atopic subjects generate enhanced levels of ROS (2, 3), which may directly injure tissues in the airways (4) and can react with eosinophil peroxidase to produce further tissue-damaging microbicidal products (1). Neutrophils are also well characterized for their ability to release O<sub>2</sub><sup>-</sup> (5). Interestingly, neutrophils from asthmatic subjects have been shown to generate more O<sub>2</sub><sup>-</sup> than those from normal individuals (6, 7). In all cell types exhibiting respiratory burst, regulated generation of O<sub>2</sub><sup>-</sup> is dependent on assembly and activation of the normally latent NADPH oxidase complex in cell membranes (8, 9).

The molecular mechanisms associated with NADPH oxidase assembly and activation have been studied in more detail in neutrophils (5, 8, 10–13) and cell-free assays (10, 14) than in eosin-

ophils (15–19). This complex is composed of five essential subunits, the membrane-bound cytochrome *b*<sub>558</sub> (a complex of two subunits, p22<sup>phox</sup> and gp91<sup>phox</sup>) which associates with cytosolic subunits Rac, p47<sup>phox</sup>, and p67<sup>phox</sup> during stimulation (8). Rac, a small monomeric GTP-binding protein of ~21 kDa, belongs to the Rho family of GTPases (20) and is bound to cytosolic Rho guanine dissociation inhibitor (RhoGDI) under basal conditions. Rac1 shares 92% amino acid homology with Rac2, and these are functionally interchangeable in their ability to activate NADPH oxidase (14, 21–23), whereas other GTPases, including K-Ras, Rap1A, Rap1B, RhoA, and Cdc42Hs, are unable to activate this complex in cell-free assays (24). Rac2 expression is limited to hemopoietic cells (25). During respiratory burst, p47<sup>phox</sup> and p67<sup>phox</sup> (in a complex with p40<sup>phox</sup>) become phosphorylated and translocate to cell membranes to bind cytochrome *b*<sub>558</sub>. Concurrent phosphorylation of a putative guanine nucleotide exchange factor induces dissociation of Rac from RhoGDI and binding of activated Rac-GTP to gp91<sup>phox</sup> and p67<sup>phox</sup> in the membrane (24).

Studies on eosinophil NADPH oxidase activation have shown strong similarities between eosinophils and neutrophils in assembly and activation of this complex. However, eosinophils generate up to 10-fold more extracellular O<sub>2</sub><sup>-</sup> than neutrophils, which may be caused by elevated expression of NADPH oxidase in eosinophils (15–17, 26, 27). Several reports have alluded to the possibility that stimulated neutrophils mainly generate O<sub>2</sub><sup>-</sup> intracellularly to assist in phagocytic killing (28, 29). However, there is a lack of direct evidence of expression and location of NADPH oxidase in human eosinophil vs neutrophil respiratory burst.

In this study, we hypothesized that eosinophils preferentially assemble NADPH oxidase in the plasma membrane to generate extracellular O<sub>2</sub><sup>-</sup>. In addition, we investigated whether human eosinophils express Rho-related GTPases, which are required for O<sub>2</sub><sup>-</sup>

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<sup>3</sup> Abbreviations used in this paper: ROS, reactive oxygen species; RhoGDI, Rho guanine dissociation inhibitor; DHR-123, dihydrorhodamine-123; MFI, mean fluorescence intensity; MBP, major basic protein; PI3K, phosphatidylinositol 3-kinase.

release through NADPH oxidase activation. Using a combination of subcellular fractionation and confocal laser scanning microscopic techniques, we describe a clear divergence in the spatial distribution of the critical oxidase regulator Rac between eosinophils and neutrophils both *in vitro* and *ex vivo*. Understanding the molecular details regulating this process and distinct patterns of regulated  $O_2^-$  release in these two cell types may contribute to development of novel and cell-specific therapeutic targets to modulate mediator secretion in asthmatic inflammation.

## Materials and Methods

### Materials

Nycodenz was purchased from Life Technologies (Burlington, Ontario, Canada). Baculovirus-generated recombinant Rac2 and rabbit polyclonal antiserum to recombinant human Rac2 were generously provided by Dr. G. M. Bokoch (The Scripps Research Institute, La Jolla, CA) (30, 31). Rabbit polyclonal Ab to RhoGDI was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-Rac Abs were purchased from Upstate Biotechnology (Lake Placid, NY) and BD Transduction Laboratories (Mississauga, Canada). Dihydrorhodamine-123 (DHR-123) was obtained from Molecular Probes (Eugene, OR). Rabbit polyclonal antisera anti-recombinant human p47<sup>phox</sup> and p67<sup>phox</sup> were kindly supplied by Dr. W. Nauseef (University of Iowa, Iowa City, IA). Mouse monoclonal anti-human p22<sup>phox</sup> and gp91<sup>phox</sup> proteins were generously provided by Dr. M. T. Quinn (Montana State University, Bozeman, MT). *Clostridium difficile* toxin B was purchased from List Biological Laboratories (Campbell, CA).

### Preparation of eosinophils and neutrophils

To isolate eosinophils, peripheral blood (100 ml) was obtained from mild atopic asthmatic and atopic nonasthmatic subjects displaying eosinophilia >2%, and who were not receiving oral corticosteroids (32, 33). Briefly, whole blood was subjected to erythrocyte sedimentation in 6% dextran, and upper phase cells were centrifuged on a single-step Ficoll (Pharmacia, Peapack, NJ) gradient before highly purified CD16<sup>+</sup> eosinophils (>99%) were isolated by negative immunomagnetic selection. For neutrophils, 50–100 ml of peripheral blood were obtained from normal subjects (except for experiments using DHR-123, in which both eosinophils and neutrophils were obtained from the same atopic donor), which was subjected to erythrocyte sedimentation in 6% dextran, followed by density centrifugation on Ficoll. Using this isolation technique, neutrophil purity usually averaged >98%.

### Measurement of $O_2^-$ release from eosinophils

Generation of extracellular  $O_2^-$  from cells in suspension was measured as previously described (19). Briefly, cells ( $1-2 \times 10^6$ ) were suspended in 1-ml microcuvets containing supplemented PBS (PBS<sup>+</sup>), pH 7.4 (with 1.2 mM MgCl<sub>2</sub>, 5 mM KCl, 0.5 mM CaCl<sub>2</sub>, 5 mM glucose, and 0.1% BSA) and 50  $\mu$ M ferricytochrome *c* at 25°C. The mixture was blanked at 550 nm in a Beckman DU 640 spectrophotometer (Beckman Instruments, Mississauga, Canada) before adding PMA at doses ranging from 1 to 1000 ng/ml. Superoxide dismutase-inhibitable OD was calculated using  $\epsilon = 2.11 \times 10^4$  M<sup>-1</sup> cm<sup>-1</sup> for reduced cytochrome *c*. To inhibit  $O_2^-$  production, *C. difficile* toxin B (40–800 ng/ml) was added to  $1 \times 10^7$  cells/ml in RPMI 1640 (Life Technologies) containing 15% FCS and incubated at 37°C for 2–20 h before treatment with PMA.

Dihydrorhodamine-123 (DHR-123, 1  $\mu$ M), a membrane-permeable probe sensitive to oxidation by ROS to produce fluorescent rhodamine-123, was incubated at 37°C for 10 min with cells ( $1-2 \times 10^6$ ) before adding 1  $\mu$ g/ml PMA for 10 min using a modification of previously published protocols (34, 35). Cells were then subjected to flow cytometric analysis (FACScan; BD Biosciences, San Jose, CA). MFI values were obtained from gated regions on dot plots for each sample.

### RT-PCR

Highly purified batches of eosinophils and neutrophils ( $\geq 99\%$ ) were subjected to total RNA extraction using a Qiagen RNeasy MiniKit (Qiagen, Mississauga, Canada) using  $2 \times 10^6$  cells per extraction (producing 0.2–1  $\mu$ g RNA) (19). Primer sequences used for detecting Rac1/2 mRNA were generated in our laboratory for Rac1 (product size, 575 bp), with forward and reverse sequences as follows: 2–24 bp, 5'-TGCAGGCCATCAAGTGTGTGGTGG-3' and (554–576 bp) 5'-CAACAGCAGGCAATTTCTCTCC-3'. Rac2-specific primers (product size, 576 bp) were (3–25 bp) 5'-GCAGGCCATCAAGTGTGTGGTGG-3' and (556–578 bp) 5'-TAGAGGAGGCTGCAGGCGCGCTT-3', respectively. Both sets of primers are

intron spanning (36, 37). Reactions were carried out in a PTC 100 Thermal Controller (M-J Research, Watertown, MA) using an annealing temperature of 57°C (Rac1-specific primers) or 56°C (Rac2-specific primers). Nonreverse-transcribed samples were included as controls.

### Western blot analysis

Samples were subjected to acrylamide gel electrophoresis and transferred to Immobilon polyvinylidene difluoride membrane blots (19). Primary mouse mAbs to Rac (1/1000), p22<sup>phox</sup> (1/1000), gp91<sup>phox</sup> (1/1000), or rabbit polyclonal Abs to Rac2 (1/500), RhoGDI (1/500), p47<sup>phox</sup> (1/1000), and p67<sup>phox</sup> (1/1000) were used before introduction of secondary Abs (1/5000 sheep anti-mouse IgG or 1/5000 donkey anti-rabbit IgG conjugated to HRP, Amersham Canada, Oakville, Canada). Chemiluminescence was developed by addition of SuperSignal substrate solution (Pierce, Rockford, IL).

### Subcellular fractionation of eosinophils and marker enzyme assays

Eosinophils were subjected to homogenization through a ball-bearing cell homogenizer (HGM Precision Engineering, Heidelberg, Germany), followed by production of postnuclear supernatant from which organelles were separated by linear density gradient (19, 38).

PMA-stimulated eosinophils ( $\geq 5 \times 10^7$  cells) were prewarmed to 37°C in 5 ml of PBS<sup>+</sup> with 250 U/ml catalase and 50 U/ml superoxide dismutase for 5 min (19). PMA was added to a final concentration of 500 ng/ml for 8 min at 37°C. The reaction was terminated by addition of 10 ml ice-cold PBS<sup>+</sup>, and cells were homogenized as described above.

Profiles of marker enzyme activities were obtained using previously reported techniques (19, 33, 38). Fractions enriched in cytosol (lactate dehydrogenase), plasma membrane/small secretory vesicles (CD9), crystalloid granules (eosinophil peroxidase), and crystalloid/small granules ( $\beta$ -hexosaminidase) were determined in supernatants and pellets using modifications of microtiter plate assays (33). Plasma membrane activity was determined by dot blot analysis with mAb to CD9 (32).

### Double labeling and confocal laser scanning microscopy

Granulocyte cytopins (50  $\mu$ l of  $0.8 \times 10^6$  cells/ml in RPMI 1640 supplemented with 20% FCS) were prepared by Cytospin 2 centrifugation (Shandon, Astmoor, Runcorn, U.K.) as previously optimized (39). Primary labeling was conducted with 20  $\mu$ g/ml mouse monoclonal anti-human Rac, specific for both Rac1 and Rac2 (mouse IgG2b; Upstate Biotechnology). Immunoreactivity to Rac was detected using 1.4  $\mu$ g/ml Rhodamine Red-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) (33, 40). Slides were double labeled with 20  $\mu$ g/ml anti-human p22<sup>phox</sup>. Bound anti-p22<sup>phox</sup> was detected by incubating 20  $\mu$ g/ml BODIPY FL-labeled goat anti-mouse IgG (Molecular Probes). Mouse IgG<sub>1</sub> and IgG2b (20  $\mu$ g/ml) were used as isotype controls (R&D Systems, Minneapolis, MN; Sigma-Aldrich, St. Louis, MO). Cells were counterstained with 4',6'-diamidino-2-phenylindole nuclear stain. Slides were mounted with 30  $\mu$ l of antibleaching agent (0.4% *n*-propyl gallate; Sigma) in glycerol-TBS (3:1) before coverslip attachment and then examined using a  $\times 40$  objective on a Zeiss confocal laser scanning microscope (Carl Zeiss Microimaging, Thornwood, NY). Images were collected and processed as described (33).

### Sputum collection and analysis

Sputum was induced from normal and well-characterized asthmatic subjects by inhalation of nebulized saline at increasing concentrations (up to 5%) (41). Atopic asthmatic subjects (5), as determined by the skin prick test, exhibited forced expiratory volume at 1 s scores of <80% of predicted values at the time of diagnosis. All asthmatics were receiving medication (inhaled corticosteroids and  $\beta$ -agonists). After inhalation of nebulized saline, subjects rinsed their mouths and then coughed sputum into sterile collection containers. Mucus plugs were manually removed and fixed immediately in 4% paraformaldehyde in PBS for 2 h. Samples were paraffin embedded via butanol in a tissue array fashion (42). For immunohistochemical analysis, samples were sectioned and stained as described above for cytopins for Rac and major basic protein (MBP) immunoreactivity. To detect MBP, 1% mouse mAb to human MBP (BMK-13, generated in-house (43)) was applied to sections. Bound BMK-13 was detected by 5  $\mu$ g/ml BODIPY FL-conjugated goat anti-mouse IgG. Slides were analyzed by confocal laser scanning microscopy on a Zeiss system, described above, and by deconvolution restoration microscopy using a DeltaVision microscope system (Applied Precision, Issaquah, WA).

### Data presentation

Enzyme activities corresponding to granule, membrane and cytosol constituents after fractionation were expressed as frequency distributions (32). Data were analyzed by ANOVA or Kruskal-Wallis statistical comparison with individual samples compared by Tukey's or Dunn's multiple comparison test.

## Results

### Extracellular vs intracellular $O_2^-$ levels generated by eosinophils and neutrophils

Eosinophils and neutrophils generated extracellular  $O_2^-$  in response to PMA stimulation, although there was a significant discrepancy in amounts of  $O_2^-$  produced from each cell type. Continuous spectrophotometric measurement of cytochrome *c* reduction in response to increasing doses of PMA (Fig. 1, A and B) revealed that the peak rate of  $O_2^-$  release was 3.4-fold higher from eosinophils than from neutrophils, similar to a value of 3.2-fold

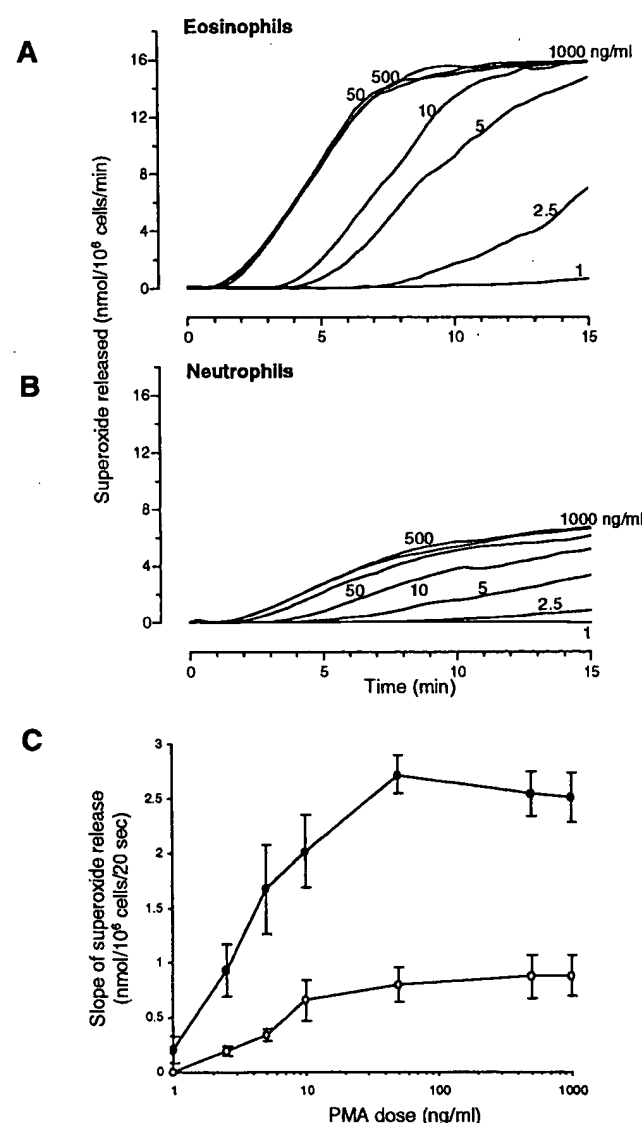
obtained previously (44) (Fig. 1C). At the maximally stimulating dose of 50 ng/ml PMA, the average peak rate of  $O_2^-$  release from eosinophils was  $15.9 \pm 0.6$  nmol  $O_2^-/10^6$  cells/min compared with  $6.2 \pm 0.7$  nmol  $O_2^-/10^6$  cells/min for neutrophils.

These values are close to those obtained for PMA-induced  $O_2^-$  production in eosinophils and neutrophils in a previous study, which reported  $13.2 \pm 1.1$  nmol  $O_2^-/10^6$  cells/min in eosinophils and  $7.2 \pm 1.3$  nmol  $O_2^-/10^6$  cells/min in neutrophils in response to 100 ng/ml PMA (27). In addition, the peak rate of  $O_2^-$  generation in eosinophils always exceeded that of neutrophils at all doses of PMA  $>1$  ng/ml (Fig. 1C). These findings support those of earlier reports (17, 27, 45). Although the response reached a plateau within 5–10 min of stimulation, the saturation appears not to be due to consumption of total cytochrome *c* in the reaction because lowering the concentration of cells did not result in loss of the plateau (data not shown).

A fluorescent probe assay coupled with flow cytometric analysis was used to determine ROS production in eosinophils and neutrophils. The membrane-permeable fluorochrome DHR-123 is unable to discriminate between intracellular and extracellular production of ROS, because it primarily reacts with  $H_2O_2$ , which is also membrane permeable and has the capacity to re-enter cells during PMA incubation before FACS analysis (46). Thus, total ROS production (both intra- and extracellular) may be measured using this fluorescent probe. Cells were incubated in the presence of DHR-123 at 37°C for 10 min before addition of 1  $\mu$ g/ml PMA for 10 min at the same temperature and then subjected to flow cytometric analysis. Using this assay, we observed an average increase of 3014 MFI U in PMA-stimulated eosinophils compared with 3857 MFI in similarly stimulated neutrophils (Table I). The increase in MFI in both cell types was significant compared with unstimulated cells ( $p < 0.01$  for eosinophils;  $p < 0.05$  for neutrophils). However, there was no significant difference in the PMA-stimulated MFI values between cell types. These data suggest that neutrophils may generate total amounts of intra- and extracellular ROS similar to those for eosinophils during respiratory burst, in contrast to previously published data (17, 27, 44, 45).

### Is Rho-related GTPase activation essential for $O_2^-$ release in eosinophils?

We determined whether Rho-related GTPases are required for NADPH oxidase activation in eosinophils by incubating cells with *C. difficile* toxin B. As shown in Fig. 2, increasing doses of toxin B reduced  $O_2^-$  generation in response to a suboptimal dose of PMA (10 ng/ml). Inhibition was significant at 400 ng/ml toxin B ( $p < 0.05$ ) and was further suppressed at 800 ng/ml (54% inhibition;  $p < 0.01$ ). No significant inhibition was detected after 2 and 16 h of incubation with toxin B at these doses (data not shown). These findings indicate that Rho-related GTP-binding proteins may be involved in regulation of PMA-induced  $O_2^-$  release in eosinophils.



**FIGURE 1.**  $O_2^-$  generation in eosinophils and neutrophils. A, Eosinophils ( $1 \times 10^6$ ) stimulated by PMA (1–1000 ng/ml); B, neutrophils ( $2 \times 10^6$ ) stimulated under similar conditions; C, slopes of  $O_2^-$  release in response to increasing doses of PMA in eosinophils (●) and neutrophils (○) in response to increasing doses of PMA during a 15-min period. Results were averaged from four to five separate experiments.

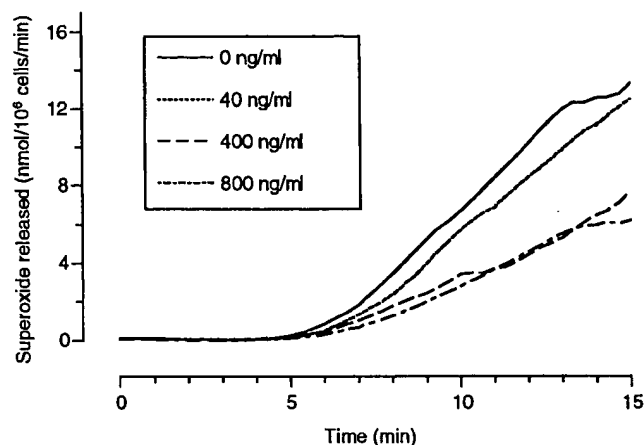
**Table I.** Measurement of MFI in eosinophils and neutrophils loaded with DHR-123 (1  $\mu$ M) and stimulated for 10 min with PMA (1  $\mu$ g/ml) at 37°C\*

	Eosinophils	Neutrophils
Unstimulated	253 $\pm$ 56	1112 $\pm$ 148
PMA stimulated (1 $\mu$ g/ml)	3267 $\pm$ 905**	4969 $\pm$ 903*

\* Values represent average MFI obtained in five separate experiments.

\*,  $p < 0.05$ .

\*\*,  $p < 0.01$ .



**FIGURE 2.** Sensitivity of  $O_2^-$  generation to toxin B incubation in eosinophils. Eosinophils ( $1 \times 10^6$ ) were incubated at  $37^\circ\text{C}$  in the presence of 0–800 ng/ml toxin B for 20–24 h and stimulated by 10 ng/ml PMA. These results represent mean  $\pm$  SEM of responses from three measurements (two different donors). Toxin B decreased maximal PMA-induced  $O_2^-$  release by 42% at 400 ng/ml ( $p < 0.05$ ) and by 53% at 800 ng/ml ( $p < 0.01$ ).

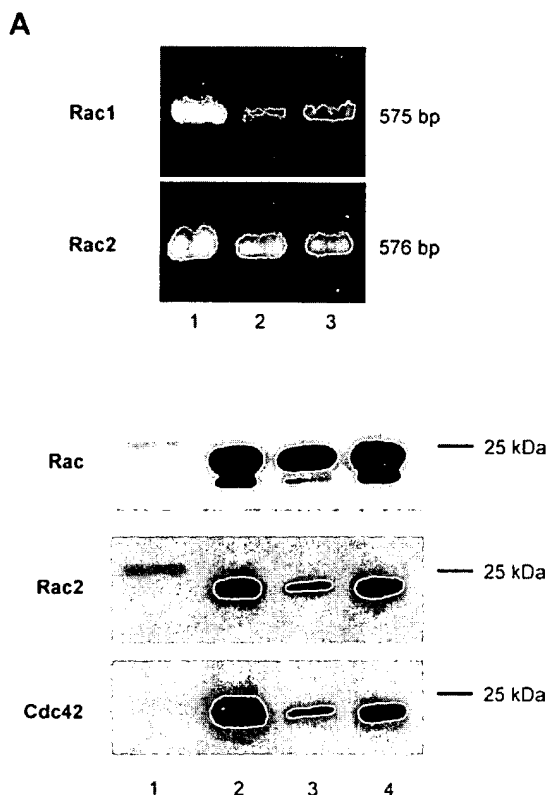
#### Eosinophil expression of Rac1, Rac2, and Cdc42 message and protein

To determine expression of Rho-related proteins in eosinophils, we generated primers detecting human Rac1 (using GenBank accession number NM\_006908.2) and Rac2 (using GenBank accession number M29871). Primers specific for Rac1 and Rac2 generated products migrating to sizes similar to those from human neutrophils (575 bp for Rac1; 576 bp for Rac2) (Fig. 3A). We also generated primers for human Cdc42. However, these were not intron spanning as the gene sequence for human Cdc42 (GenBank accession number M35543) does not contain any introns (47). Consequently, we obtained PCR products for Cdc42 in non-reverse-transcribed control RNA samples, indicating contamination by genomic DNA (data not shown).

Western blot analysis of whole cell homogenates using Abs raised against human Rac1, Rac2, and Cdc42 demonstrated that Rac and Cdc42 proteins were expressed in eosinophils (Fig. 3B). We were unable to determine whether eosinophils express Rac2 in preference to Rac1, since currently available Abs do not specifically recognize Rac1.

#### Translocation of NADPH oxidase components to cell membrane fractions occurs in parallel with $O_2^-$ generation

Membrane translocation of cytosolic NADPH oxidase components (Rac1/2,  $p47^{phox}$ , and  $p67^{phox}$ ) has been previously shown to correlate with  $O_2^-$  production in neutrophils (30, 31) and guinea pig eosinophils (18, 19). To determine whether similar translocation occurred in human eosinophils,  $5 \times 10^7$  eosinophils were subjected to subcellular fractionation and immunoblot analysis before and after stimulation with PMA. Fig. 4 shows the profiles of marker enzyme activities and corresponding immunoreactivities for these components. Positions of intracellular organelles determined by marker enzyme assays before and after PMA stimulation were unchanged (data not shown), indicating that PMA did not alter organelle densities. Before stimulation, Rac, RhoGDI,  $p47^{phox}$ , and  $p67^{phox}$  were predominantly expressed in cytosolic fractions, whereas the cytochrome  $b_{558}$  subunits,  $p22^{phox}$  and  $gp91^{phox}$ , were localized to plasma membrane-rich fractions. After 8 min of stimulation with PMA (500 ng/ml),  $p47^{phox}$  and  $p67^{phox}$ , but not RhoGDI, translocated to fractions containing cytochrome



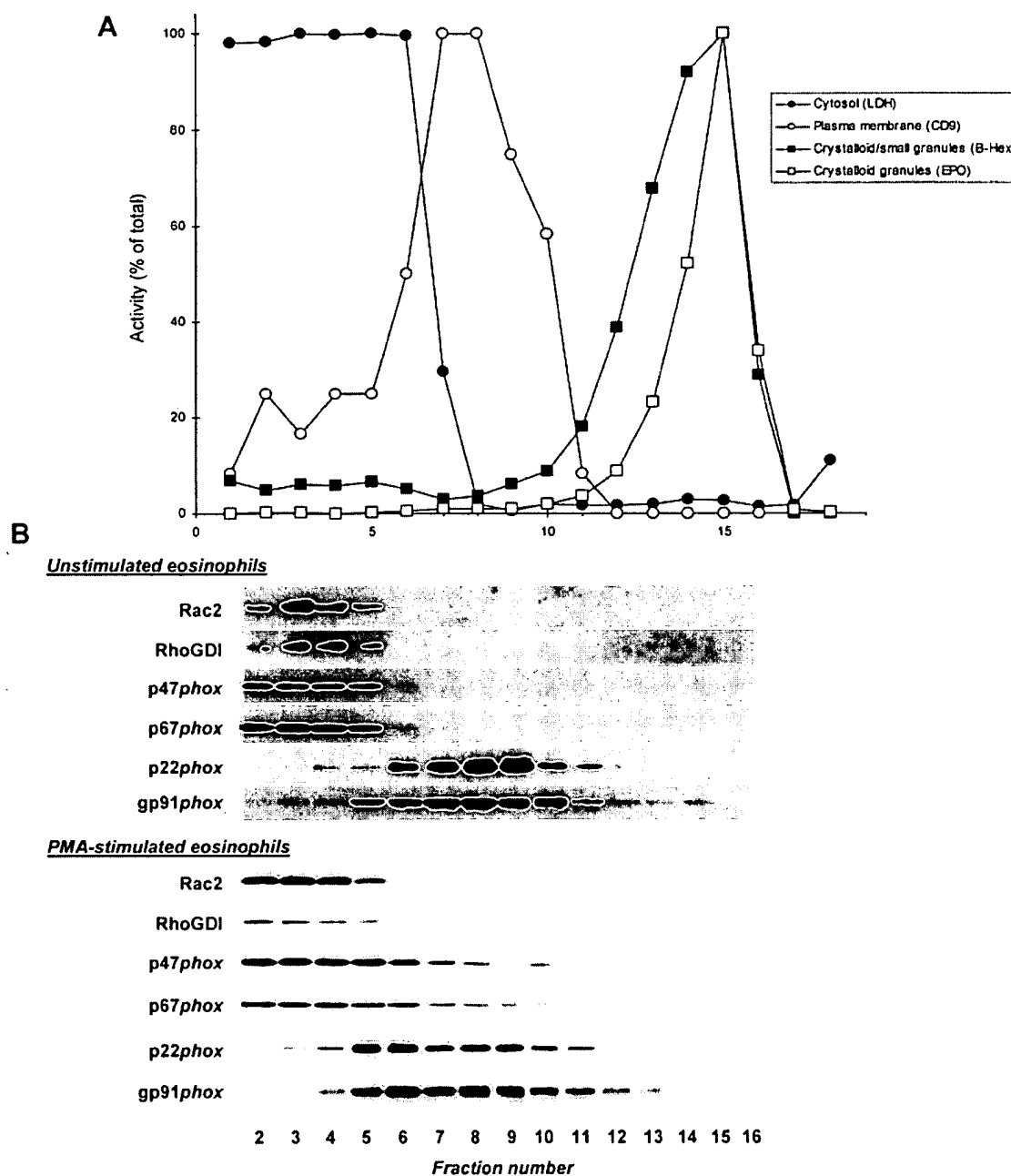
**FIGURE 3.** Expression of Rho GTPases in eosinophils. A, RT-PCR of RNA samples from guinea pig peritoneal eosinophils (lane 1), human eosinophils (lane 2), and human neutrophils (lane 3). RNA samples were treated with DNase before RT-PCR analysis to remove genomic contamination. Results are representative of five preparations. B, Immunoblot analysis of Rac1/2 and Cdc42 expression in eosinophils. Samples loaded were baculovirus-generated recombinant Rac2, 0.1  $\mu\text{g}$  (lane 1), guinea pig macrophages (78%), 20  $\mu\text{g}$  (lane 2), human eosinophils (97%), 20  $\mu\text{g}$  (lane 3), and human neutrophils (99%), 20  $\mu\text{g}$  (lane 4). Abs to Rac (BD Transduction Labs) and Rac2 (Dr. G. M. Bokoch) were used. Baculovirus-generated Rac2 protein migrated at a higher apparent  $M_r$  than cell samples due to the presence of additional amino acids upstream of the Rac2 sequence. The mouse monoclonal anti-human Rac1 from BD Transduction Labs did not discriminate between Rac1 and Rac2. Similar results were obtained with an Ab to human Rac from Upstate Biotechnology (results not shown). Results are representative of three separate experiments.

$b_{558}$  immunoreactivity, although no discernable Rac translocation occurred. A small shift in immunoreactivities for  $p22^{phox}$  and  $gp91^{phox}$  toward lower density fractions (fractions 5 and 6) was observed in PMA-stimulated cells, suggesting that cytochrome  $b_{558}$  may be present in a population of small secretory vesicles that fuse with the plasma membrane on activation.

#### Double labeling of Rac and $p22^{phox}$ demonstrates divergence in eosinophil and neutrophil NADPH oxidase assembly

Intracellular sites of NADPH oxidase assembly have not yet been determined in eosinophils, although neutrophils are well known for their expression of oxidase components in specific granules (48, 49). We sought to determine whether eosinophils translocate Rac to cell membranes in correlation with extracellular  $O_2^-$  generation.

Rac immunofluorescence was cytosolically distributed in unstimulated eosinophils (Fig. 5A), whereas  $p22^{phox}$  was membrane associated (Fig. 5B) with some intracellular staining. Overlaid images showed little colocalization between Rac and  $p22^{phox}$  under



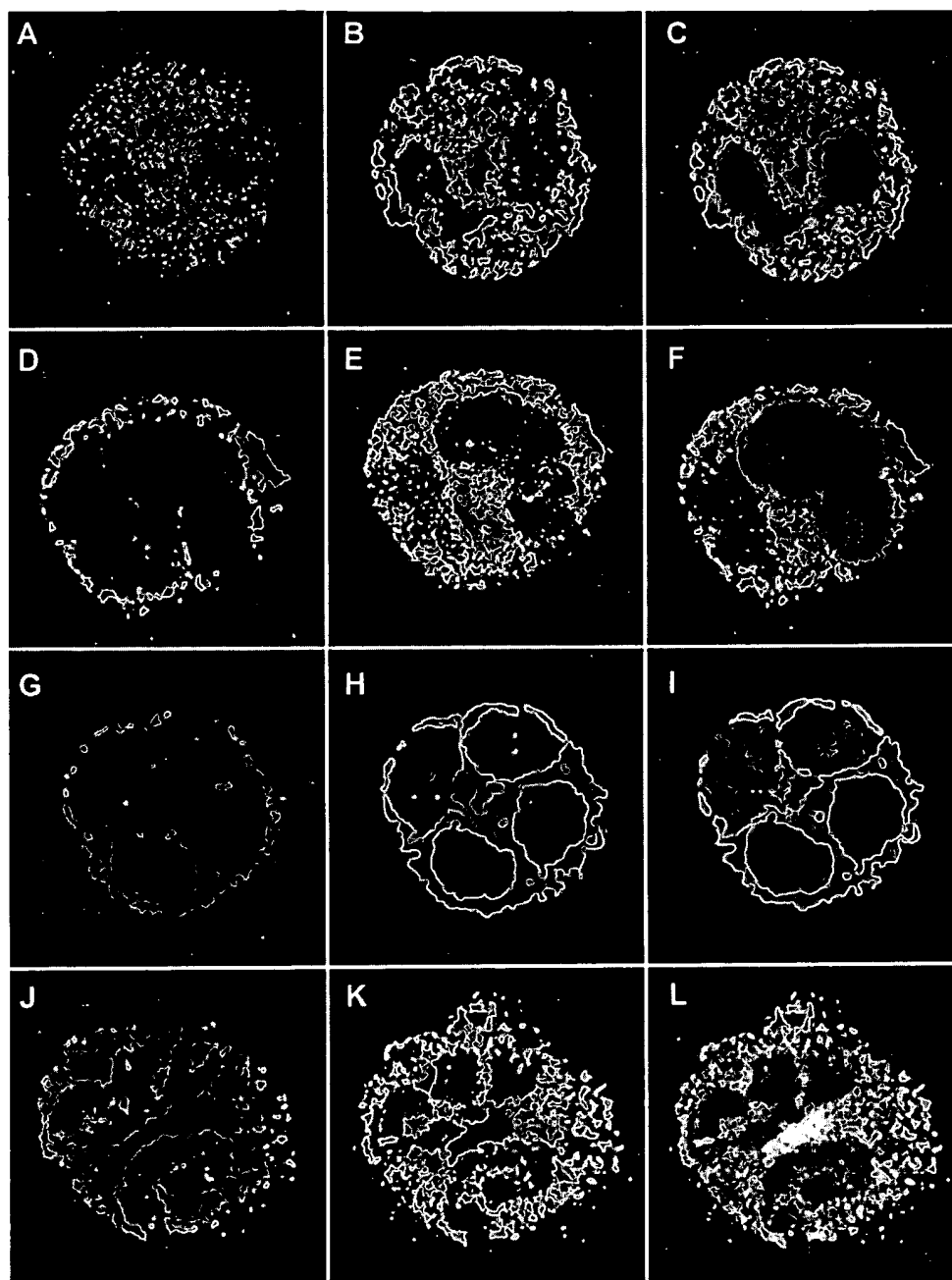
**FIGURE 4.** Subcellular fractionation of eosinophils and immunoblot analysis of NADPH oxidase components. **A**, Marker enzyme profiles of eosinophils after subcellular fractionation, indicating locations of cytosol (lactate dehydrogenase; LDH), plasma membrane/small secretory vesicles (CD9), and crystalloid granules (EPO); **B**, Immunoblot analysis of Rac2, RhoGDI, p47<sup>phox</sup>, p67<sup>phox</sup>, p22<sup>phox</sup>, and gp91<sup>phox</sup> expression in unstimulated and PMA-stimulated cells. Cells were stimulated with 500 ng/ml PMA for 8 min before termination of reaction.

basal conditions (Fig. 5C). Stimulation of O<sub>2</sub><sup>-</sup> release in eosinophils resulted in a pronounced translocation of Rac from the cytoplasm to the cell periphery (Fig. 5D), to colocalize with membrane p22<sup>phox</sup> immunofluorescence (Fig. 5F).

Resting neutrophils exhibited a pattern of Rac and p22<sup>phox</sup> immunofluorescence similar to that of eosinophils (Fig. 5, G-I). However, stimulated neutrophils showed a distinct translocation of Rac from that of eosinophils, in which increased intracellular Rac staining was detected which colocalized with the p22<sup>phox</sup> label (Fig. 5, J and L). These findings suggest that, unlike eosinophils, activated NADPH oxidase predominantly assembled at intracellular sites in neutrophils.

#### *Eosinophils and neutrophils in asthmatic sputum samples exhibit distinct patterns of Rac distribution*

We examined eosinophils in sputum from atopic asthmatics to examine patterns of Rac staining *ex vivo*. We were surprised to find that granulocytes in sputum exhibited Rac staining similar to those stimulated *in vitro*. As shown in Fig. 6, B-F, morphologically intact MBP<sup>+</sup> eosinophils in asthmatic sputum samples exhibited peripheral immunostaining for Rac along cell membranes, analogous to that found in PMA-stimulated eosinophils (Fig. 5D). In contrast, MBP<sup>+</sup> eosinophils in normal sputum samples, which were rare, expressed low levels of cytosolic Rac (Fig. 6G), comparable with unstimulated



**FIGURE 5.** Confocal analysis of respiratory burst in eosinophils and neutrophils. Cells were double labeled for Rac and p22<sup>phox</sup>, followed by DAPI nuclear counterstain (blue). Rac immunofluorescence (A, D, G, and J), shown in red, was overlaid with green p22<sup>phox</sup> immunofluorescence (B, E, H, and K) to determine colocalization as indicated by yellow-orange color (C, F, I, and L). Anti-Rac from Upstate Biotechnology was used in these experiments. A–C, Resting eosinophils; D–F, PMA-stimulated (500 ng/ml) eosinophils. Neutrophils, shown in G–I, were compared with PMA-stimulated cells (J–L). Original magnification,  $\times 40$ .

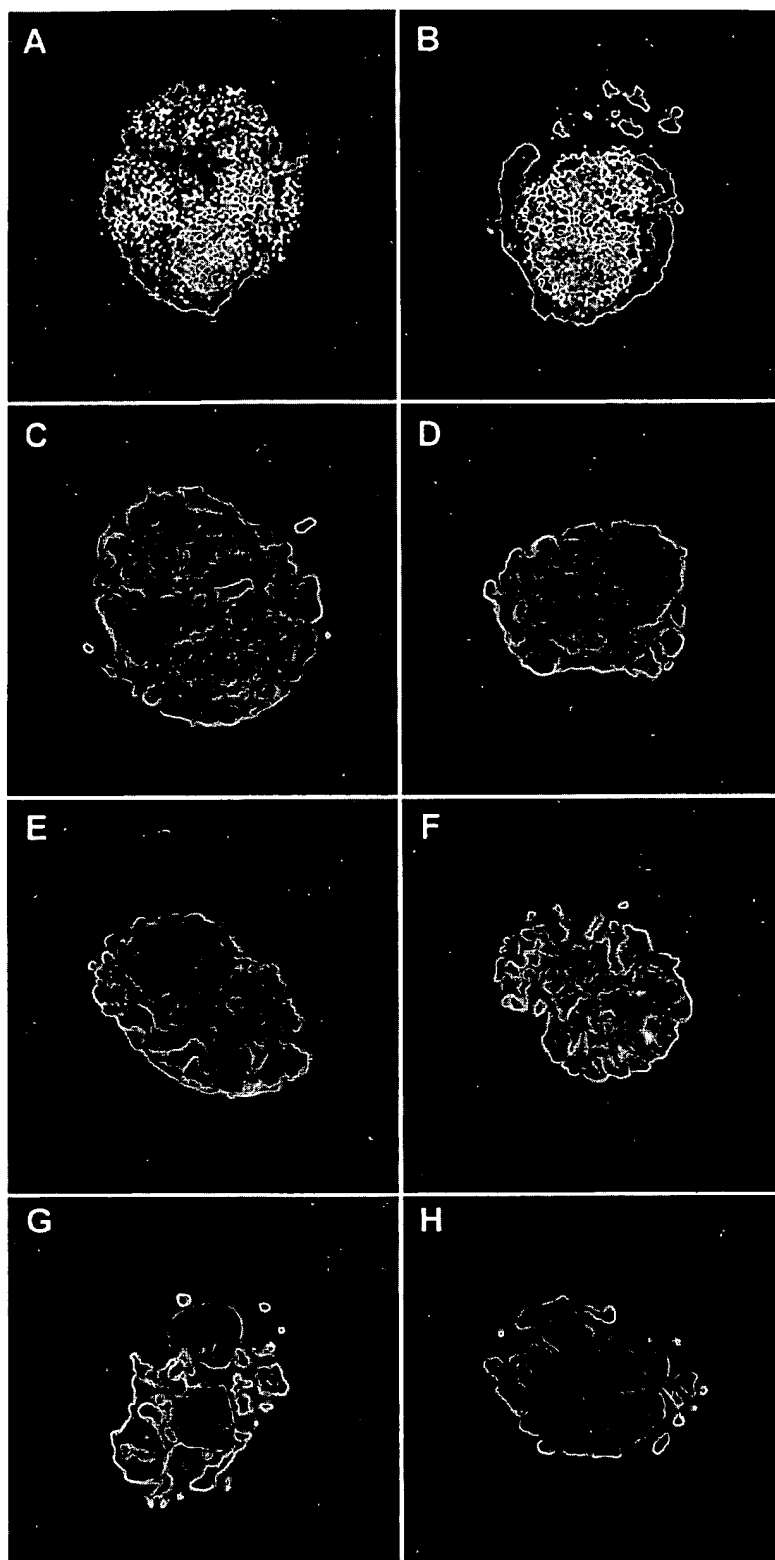
peripheral blood eosinophils (Fig. 5A). Moreover, MBP<sup>+</sup> polymorphonuclear neutrophils in asthmatic sputum, identified based on their multilobular nuclear morphology using 4',6'-diamidino-2-phenylindole nuclear counterstaining, displayed substantial intracellular Rac staining (Fig. 6H), similar to those stimulated in vitro with PMA (Fig. 5J).

## Discussion

Translocation and assembly of NADPH oxidase are essential for regulated O<sub>2</sub><sup>•−</sup> generation in phagocytes. Activation of this complex is critically dependent on receptor stimulation of intracellular regulatory Rho-related GTPases, principally Rac1 or its homolog

Rac2. In this study, PMA-induced O<sub>2</sub><sup>•−</sup> generation in eosinophils was shown to require Rac stimulation of NADPH oxidase, as demonstrated by its sensitivity to toxin B inhibition. Although toxin B inhibits Rho, Rac, and Cdc42 by monoglucosylation at Thr<sup>37</sup> or Thr<sup>35</sup> (50), it is likely that the inhibitory effect of toxin B on O<sub>2</sub><sup>•−</sup> production in eosinophils was mediated through blockade of Rac1 or Rac2. This is based on findings from cell-free assays that demonstrated that only Rac1 and Rac2, and not Rho or Cdc42, were able to activate NADPH oxidase in reconstituted lipid bilayers (24).

In addition, human eosinophils were shown to express both Rac1 and Rac2 mRNA, although only Rac2 protein could be positively identified by Western blot analysis. This is similar to guinea



**FIGURE 6.** Confocal and deconvolution restoration microscopic analysis of eosinophils and neutrophils in sputum samples. Confocal images are shown for sputum eosinophils from normal (A) and asthmatic (B) subjects after labeling with anti-Rac, shown in red fluorescence, and MBP, as indicated by green. Deconvolution restoration microscopy was also conducted on eosinophils (C–F) and neutrophils (H) from asthmatic sputum. All panels were produced from combined images of double labeling. A sputum eosinophil from a normal donor (G) is shown for comparison. Original magnification,  $\times 63$ . Sections of asthmatic sputum shown here are representative of samples obtained from five atopic asthmatics.

pig eosinophils, which may produce both isoforms (18, 19). Human neutrophils, in contrast, have been shown to predominantly express Rac2 protein (21, 31). Rac2 binds with a 6-fold higher affinity with p67<sup>phox</sup> than Rac1 in two-hybrid assays, suggesting that it may be a more effective inducer of oxidase activity than Rac1 (51). Previous studies have demonstrated expression of p40<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup>, and cytochrome *b*<sub>558</sub> in human eosino-

phils (15, 17, 52–55) but did not show expression of Rho-related GTPases. Eosinophils may preferentially express Rac2 rather than Rac1 based on a shared hemopoietic lineage with neutrophils, although we were unable to determine Rac1 expression based on our Western blot data.

Translocation of oxidase components during respiratory burst has not previously been demonstrated in human eosinophils. In our

study, translocation of cytosolic p47<sup>phox</sup> and p67<sup>phox</sup> to plasma membrane at 8 min of PMA stimulation in eosinophils was similar to earlier observations on guinea pig eosinophils (18, 19) and correlated with observations in neutrophils (30). This study demonstrated that p47<sup>phox</sup> and p67<sup>phox</sup> translocated to the membrane during respiratory burst on an equimolar basis with Rac. The majority of cellular cytochrome *b*<sub>558</sub> is localized to specific granule membranes in neutrophils, which is transferred to plasma or phagosomal membranes on activation (48, 49, 56). Cytosolic p47<sup>phox</sup> and p67<sup>phox</sup> associate with cytochrome *b*<sub>558</sub> after phosphorylation of specific serine/threonine sites (8). These bind through Src homology 3 and pleckstrin homology domains to allow association of Rac to plasma membrane and p67<sup>phox</sup>. Collectively, these subunits work to initiate electron transfer from NADPH to flavin adenine di-nucleotide through cytochrome *b*<sub>558</sub>, ultimately resulting in formation of O<sub>2</sub><sup>-</sup> from O<sub>2</sub> on the external or luminal surface of the membrane (8).

The mobilization and assembly of NADPH oxidase to cell membranes in human eosinophils correlated with maximal O<sub>2</sub><sup>-</sup> generation as determined by cytochrome *c* reduction assays. This procedure measures only extracellular production of O<sub>2</sub><sup>-</sup>, since O<sub>2</sub><sup>-</sup> and cytochrome *c* are membrane impermeable. Previous studies using immunoblot analysis have demonstrated that eosinophils produce more NADPH oxidase than neutrophils, which was thought to explain why eosinophils generate more O<sub>2</sub><sup>-</sup> than neutrophils (15, 17, 18). However, the discrepancy in O<sub>2</sub><sup>-</sup> production between eosinophils and neutrophils may also be attributable to neutrophils preferentially generating O<sub>2</sub><sup>-</sup> inside cells. We tested this notion by stimulating cells with PMA in the presence of DHR-123, which can detect intracellular ROS production by reacting with H<sub>2</sub>O<sub>2</sub> and forming a fluorescent product inside the cells (46). Total ROS production was found to be equivalent in PMA-stimulated neutrophils and eosinophils using DHR-123. These findings suggest that neutrophils preferentially generate O<sub>2</sub><sup>-</sup> intracellularly, which may partially account for the discrepancy in cytochrome *c* measurements of O<sub>2</sub><sup>-</sup> release from eosinophils and neutrophils.

The confocal data in this study supported the possibility that eosinophils generate most of their O<sub>2</sub><sup>-</sup> extracellularly in correlation with the translocation of Rac to the cell membrane. We observed by confocal microscopy that, in striking contrast to neutrophils, eosinophils preferentially translocated Rac to the plasma membrane rather than intracellular sites following PMA stimulation. Neutrophils did not appear to translocate Rac2 to plasma membrane, and instead directed Rac2 to intracellular sites in association with p22<sup>phox</sup> of PMA stimulation. Interestingly, eosinophils are able to phagocytose extracellular *Escherichia coli* and *Staphylococcus aureus* but are unable to kill these as efficiently as neutrophils (57), which was believed to correlate with an inability to modify amino acids through the peroxidase-H<sub>2</sub>O<sub>2</sub>-Cl<sup>-</sup> system. Our findings indicate that eosinophils may be less efficient at killing due to the lack of significant intracellular O<sub>2</sub><sup>-</sup> production.

Oxidase assembly may occur in two distinct pools in the neutrophil which are regulated by different pathways. The mechanism of action of PMA is not well understood, although it is frequently assumed to activate NADPH oxidase through protein kinase C. Wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3K), has been shown to block only intracellular production of O<sub>2</sub><sup>-</sup> and not extracellular release induced by PMA (58). This indicates that a PI3K-dependent pathway may activate only intracellular O<sub>2</sub><sup>-</sup> production in neutrophils, whereas a PI3K-independent pathway regulates extracellular release of O<sub>2</sub><sup>-</sup>. In contrast, the effect of wortmannin on eosinophil respiratory burst is not well understood. Thus, whereas eotaxin-induced O<sub>2</sub><sup>-</sup> release was inhibited by wortmannin in human eosinophils, it was without effect on leukotriene

B<sub>4</sub>-induced respiratory burst in guinea pig eosinophils (1). It remains to be determined whether eosinophils utilize a PI3K-independent pathway in PMA-induced oxidase activation to release O<sub>2</sub><sup>-</sup> extracellularly.

We did not detect significant expression of oxidase components in eosinophil crystalloid granule-enriched subcellular fractions, but rather in plasma membrane/light membrane fractions (including small secretory vesicles; Fig. 4), which supports the findings of Calafat et al. (53). NADPH oxidase activation is therefore unlikely to involve crystalloid granules in eosinophils. This is in contrast to neutrophils, which express cytochrome *b*<sub>558</sub> in specific granules (48, 49, 53, 59). The majority of NADPH oxidase activity in neutrophils undergoing arachidonate- or PMA-induced respiratory burst was found to localize to specific granules (58, 60). The function of cytochrome *b*<sub>558</sub> expression in neutrophil-specific granules is thought to be associated with NADPH oxidase activation after fusion of the specific granules with newly phagocytosed particles, with the purpose of carrying out oxygen-dependent intracellular killing of phagocytosable microorganisms (24). The lack of expression of NADPH oxidase components on crystalloid granules lends further support to the suggestion that eosinophils do not generate significant intracellular O<sub>2</sub><sup>-</sup> during respiratory burst and instead directs O<sub>2</sub><sup>-</sup> towards extracellular regions. Although translocation of Rac to the cell membrane was not evident in Western blot analysis, it was detectable in confocal microscopy analysis, suggesting that the latter technique may be substantially more sensitive to translocation events than Western blot. Even when Rac2 translocation was shown in PMA-stimulated guinea pig eosinophils in our previous report (19), the quantity translocated was barely detectable by immunoblot analysis.

The distinct pattern of Rac immunoreactivity in activated eosinophils in vitro was observed in ex vivo sputum samples from asthmatic patients. Rac immunofluorescence in normal sputum eosinophils was less than that of asthmatic cells and did not exhibit a peripheral membrane pattern. In contrast, sputum eosinophils from asthmatic patients exhibited intense Rac immunofluorescence around the cell membrane, suggesting that these cells were stimulated and were actively releasing O<sub>2</sub><sup>-</sup> into the tissues and airways. These observations indicate that sputum eosinophils in unstable asthma may be activated by in vivo stimuli to produce extracellular O<sub>2</sub><sup>-</sup> and contribute to oxidant-mediated tissue injury. These novel observations indicate that eosinophil respiratory burst may be important in the pathogenesis of asthma.

In conclusion, eosinophils appear to assemble NADPH oxidase similarly to neutrophils at the level of molecular complex formation, whereas intracellular distribution of NADPH oxidase may differ significantly between these two cell types. This divergence, reflected in a predominantly plasma membrane association of Rac with p22<sup>phox</sup> in eosinophils vs a mainly intracellular location in neutrophils, may parallel distinct functional roles that these two cell types have in innate immunity. Thus, our findings suggest that sputum eosinophils from individuals with unstable asthma may be activated to produce more extracellular O<sub>2</sub><sup>-</sup> than neutrophils, with the potential to induce tissue damage and contribute to the pathogenesis of this disease. Neutrophils may produce comparatively less extracellular O<sub>2</sub><sup>-</sup> in sputum from these individuals. The differential manner of NADPH oxidase assembly in these cells may have important implications for determining the activation status of airway eosinophils and, ultimately, treatment of oxidant-mediated tissue injury in asthma.

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